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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)
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MARTIN J. PAGE)
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Serial No. 08/155,864)
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Filed: November 23, 1993) Group Art Unit: 1815
)
For: ANTIBODY PRODUCTION)

Declaration

Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Dear Sir:

I, Robert Lifely, declare that:

1. I received my B.Sc. in chemistry and mathematics from Royal Holloway College, University of London. I received my Ph.D. in microbiological chemistry from the University of Newcastle in 1978.

I am a Senior Research Scientist at Wellcome Research Laboratories, located in Beckenham, Kent, England, a position I have held since 1985. From 1978-1985 I was a Research Scientist at Wellcome Research Laboratories. In my time at Wellcome, my research has been multidisciplinary and includes chemistry, immunology, cell biology and microbiology. My work has been focused in the areas of vaccines and, for the last five years, monoclonal antibodies. A copy of my C.V. is attached hereto as Exhibit A.

2. I have reviewed and am familiar with the above-referenced patent application. This application describes the balanced expression of the heavy and light chains of a recombinant antibody from Chinese hamster ovary (CHO) cells and the use of these CHO-glycosylated antibodies in medical therapy to treat various disorders. Specifically, the antibodies can be used to treat T-cell mediated disorders, such as severe vasculitis, rheumatoid arthritis and systemic lupus, and autoimmune disorders, such as multiple sclerosis, graft vs. host disease, juvenile onset diabetes, Sjogrens' disease, thyroid disease, myasthenia gravis, transplant rejection and asthma. The antibodies also can be used to treat certain cancers, such as Non-Hodgkins lymphoma and multiple myeloma, and infectious diseases, such as HIV and herpes.

3. Antibodies produced in myeloma cells have been used to treat various diseases and disorders. It was not obvious from data involving the use of myeloma-produced antibodies to treat disease that antibodies produced in CHO cells also would be effective in medical therapy.

4. The scientific literature provides significant evidence that differences in the glycosylation of an antibody can affect its *in vitro* biological activity through altered Fc receptor binding and/or complement activation. See, for example, Nose, M. and H. Wigzell, *Proc. Natl. Acad. Sci. USA* 80:6632 (1983); Leatherbarrow, R.J., et al. *Mol. Immunol.* 22:407 (1985); and Rademacher, T.W., et al. *Ann. Rev. Biochem.* 57:785 (1988), copies

of which are enclosed as exhibits B-1, B-2 and B-3, respectively. Circulatory lifetime, immunogenicity and antigenicity of the antibody *in vivo* also can be dependent upon glycosylation of the molecule. See Goochee, C.F. et al. *Biotechnology* 8:421 (1990), a copy of which is enclosed as Exhibit B-4.

5. Several factors are known to be able to influence glycosylation. Species-, tissue- and cell-type all can influence the way that glycosylation occurs. See Goochee, C.F., et al. above.

6. CAMPATH-1H, a humanized IgG_1 monoclonal antibody directed against the CDw52 antigen present on the cell surface of the majority of normal and malignant lymphocytes, has been expressed in a rat myeloma cell line (Reichmann, L., et al. *Nature* 332:323 (1988), a copy of which is enclosed as Exhibit B-5) and used to treat successfully patients with lymphoproliferative disorders. Hale, G. et al., *The Lancet* 2:1394 (1988), a copy of which is provided as Exhibit B-6. The production of this antibody in a CHO cell is described in detail in the present application. The myeloma-derived and CHO-derived CAMPATH-1H were expected to differ in glycosylation, due to differences in species-, tissue- and cell-type.

7. The myeloma-derived and CHO-derived CAMPATH-1H have been compared on the basis of oligosaccharide composition and biological activity as measured by ELISA, antigen binding assays, complement mediated lysis (CML) and antibody dependent cell-mediated cytotoxicity (ADCC).

a. oligosaccharide mapping

The intact oligosaccharide chains were released from the CAMPATH-1H protein backbone enzymatically using glycopeptidase-F. The oligosaccharides were analyzed by two different chromatographic techniques, reverse phase (RP) HPLC and high performance anion exchange chromatography (HPAEC).

To facilitate RP-HPLC, with conventional UV detection, oligosaccharides were first derivatized with 1-phenyl-3-methyl-2-pyrazolin-5-one (PMP). A number of standard oligosaccharides were similarly derivatized to enable approximate identification of the types of structures present from comparison of retention times.

HPAEC does not require the oligosaccharides to be derivatized, but works by "on column" ionization of the hydroxyl groups, by means of a high pH eluate. The oligosaccharides then can be separated as anions in a conventional ion exchange mechanism; detection was achieved using a pulsed amperometric detector (PAD). Comparison with standard oligosaccharides allowed an indication of the types of structures involved.

The results, as shown in Figures 1 and 2, attached, indicate a clear difference in the oligosaccharide composition of CHO-derived CAMPATH-1H and myeloma-derived CAMPATH-1H.

b. biological activity

The ELISA carried out was a capture ELISA in which goat anti-human IgG (H+L) was coated onto plates and captured CAMPATH-1H was detected with peroxidase-conjugated goat anti-human IgG (gamma chain specific).

Two assays were used for measuring the ability of CAMPATH-1H to bind to the CDw52 antigen. The first was a solid phase radioassay that utilized a T-cell line (HUT-78), expressing the antigen, coated onto microtitre plates, wherein bound CAMPATH-1H was detected with ^{125}I-protein A. The second assay was an ELISA that substituted HUT-78 cells with the soluble purified antigen. Bound CAMPATH-1H was detected with alkaline phosphatase-conjugated anti-human IgG (gamma chain specific).

The CML assay was based on lysis of ^{51}Cr-labelled K-422 cells (a B-cell non-Hodgkins lymphoma cell line expressing the CDw52 antigen) with CAMPATH-1H using normal human serum as a complement source.

To carry out the ADCC, peripheral blood mononuclear cells (PBML) were used as effectors. The ADCC-antibody dependent cellular cytotoxicity assay was carried out as follows:

Wien 133 cells were pelleted and the pellet incubated with 100 μl Cr^{51}-sodium chromate (1mCi/ml) for 1 hour at 37° C (the cells were suspended by flick mixing cells 20 minutes). The cells were washed three times in serum containing medium and resuspended to 2 x 10^5/ml. Aliquots of 50 μl (i.e., 10^4 cells)

were incubated with 50 μl of various antibody dilutions for one and one half hours at 37° C in microtitre plates.

Effector cells were isolated from human blood by separation on lymphoprep (Nycomed). The resulting peripheral blood mononuclear cells (PBMC) were washed three times in serum containing medium and resuspended at either 2.5 x 10^6 ml or higher to give final effector:target ratios of at least 25:1. The PBMC were aliquoted onto the pretreated target above at 100 μl/well and the plates were spun briefly at 1.5k rpm to settle the cells.

The plates then were left at 37° C for 5 hours. Control cells were included in the assay and were either the targets incubated in medium alone (spontaneous release) or the targets incubated in 1% triton (maximum release).

100μl aliquots from each well were counted and the specific release calculate using the following formula:

$$\% \text{ specific release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 10^6$$

For the tests, purified CHO and myeloma antibodies were adjusted to a protein concentration of 1.75 mg/ml by A_{280} and by a BCA protein assay. The results of the tests are presented in Table 1 and Figure 3, attached. Both antibodies were of apparent high purity, as judged by the ELISA for antibody concentration.

Furthermore, no significant differences in biological activity of the two preparations were observed in the T-cell/soluble antigen binding and complement mediated lysis assays.

The ADCC assay, however, showed higher activity for the myeloma-derived CAMPATH-1H preparation over the CHO-derived product, which ranged from a two-to ten-fold difference, dependent upon the effector cell (PBMC) donor. This is illustrated in Figure 4.

8. In view of these findings regarding oligosaccharide composition and biological activity, it was very uncertain whether the CHO-produced antibody would be effective in treating patients.

9. The CHO-glycosylated antibody was administered to patients suffering from life-threatening autoimmune systemic vasculitis or lymphoma as reported in Lim, S. et al., *The Lancet* 341:432 (February 13, 1993); Lockwood, C.M. et al., *The Lancet* 341:1620 (June 26, 1993); and Poynton, C.H. et al., *The Lancet* 341:1037 (April 17, 1993), copies of which are provided herewith as Exhibits B-7, B-8 and B-9, respectively. Lockwood et al. report that the CHO-glycosylated Ab provided "substantial and sustained benefit" in three of the four patients when administered alone and in the fourth when administered in combination with thalidomide. Lim et al. provide that the administration of the antibody to a patient suffering from non-Hodgkin lymphoma which had proved to be resistant to chemotherapy and interferon resulted in a good response in blood, bone marrow

and spleen. They state that although complete remission did not result, the therapy did provide a substantial cytoreduction of lymphoma cells from bone marrow. Poynton et al. administered the antibody to four patients with resistant non-Hodgkin lymphoma. They reported that their patients suffered more adverse side effects than had been experienced by Lim's patient, but agreed that the antibody constitutes "an exciting new treatment of resistant non-Hodgkin lymphoma.".

10. The administration of a CHO-glycosylated anti-CDw52 antibody also has been shown to be effective therapy for persons suffering from multiple sclerosis. PCT application PCT/GB92/02252, published June 10, 1993, discloses and claims a method for treating multiple sclerosis through the administration of an antibody recognizing the CDw52 antigen. A copy of this application is provided as Exhibit B-10.

The application provides a case history in which a woman in her mid-forties was diagnosed as suffering from MS. She initially was treated with steroids, but her condition continued to worsen and the disease was judged to have entered a chronic progressive phase. She was given CHO-glycosylated CAMPATH-1H in 10 intravenous doses. (The application states on page 5 that the antibody was produced by expression in a recombinant CHO cell line.) As the application provides, according to evaluations of the patient one and two months following the introduction of the antibody therapy, the patient showed "improvement in the progression, disability and relapse assessment categories." The

application further states that the treatment course was well-tolerated and that 18 months after the treatment, the patient's improved condition had been maintained.

11. CHO-glycosylated anti-CDw52 antibodies also have been shown to have a therapeutic effect when administered to persons suffering from rheumatoid arthritis. Published application PCT/GB92/01884, a copy of which is provided as Exhibit B-11, summarizes the results of several case histories. As the application makes clear on page 5, the anti-CDw52 antibody used in case study #1 was produced in rat Y-0 myeloma cells and the antibody used in all other case studies reported was produced by expression in a recombinant CHO cell line.

The patient described in case study #2 was a woman in her twenties who suffered from a severe case of atypical Still's disease, a variant of rheumatoid arthritis which occurs in younger patients. A number of conventional therapies were tried, with at best only limited effect, and the patient required lengthy hospitalizations. She was administered CAMPATH-1H as described in the application. The treatment resulted in dramatic improvements in her condition and the remission lasted for nine months. Following a relapse, she received a combination therapy of CAMPATH-1H and an anti-CD4 antibody. When this had no sustained effect, a plasma exchange was carried out, followed by further treatment with the two antibodies, this time with good effect. The patient also received small doses of steroids, and four months later was in stable remission.

Eight other case studies are reported in the application in which the patients received CHO-glycosylated CAMPATH-1H. Each patient suffered actively from arthritis, and conventional therapies had proved to be unsuccessful. The CAMPATH-1H was administered as described in the application. Seven of the eight patients showed an "impressive" initial response to the therapy, as shown by reduction in joint swelling and improvement in thermography. Each patient enjoyed a remission which, with one exception, lasted at least two and a half months and, in one case lasted at least six months.

12. Attached hereto and marked as Exhibit B-12 is a page from a report written by Dr. Geoff Hale of the MRC/Wellcome Therapeutic Antibody Centre in Cambridge, England. In his summary of initial clinical data, Dr. Hale notes that the researchers at the Centre had doubted whether the CHO-glycosylated antibody would be effective *in vivo*. As Dr. Hale reports, however, the patient experienced a marked clinical improvement, lymphocytes were cleared, opiates which had been administered to control pain could be withdrawn and the patient was able to walk again.

Subject matter not pertaining to the clinical studies has been deleted from the attached page of the report.

13. In view of the state of the art and our own experimental findings regarding oligosaccharide composition and biological activity, the clinical results obtained by these researchers were surprising. It was not obvious at the time this

invention was made that CHO-glycosylated antibodies would be useful for medical therapy.

14. In view of the *in vivo* data showing the efficacy of CHO-glycosylated CAMPATH-1H antibodies to treat various disorders, I would expect that other antibodies produced in CHO cells also would have similar and appropriate glycosylation. Inasmuch as the glycosyl transferases present in the CHO cell lines would be the same regardless of the antibody being produced under similar cell culture environmental conditions, one of ordinary skill in the art would expect these enzymes to glycosylate any antibody in a similar manner.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

R. Lifely

Robert Lifely

6. 4. 94

Date

Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6632–6636, November 1983
Immunology

EXHIBIT E-1

Biological significance of carbohydrate chains on monoclonal antibodies

tunicamycin/Fc receptor/complement/protein A/immunocomplex

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Communicated by George Klein, June 27, 1983

ABSTRACT **We have prepared monoclonal hapten-specific mouse IgG2b antibodies depleted of asparagine-linked carbohydrate chains by treating the hybridoma cells with tunicamycin. The carbohydrate-deficient antibodies behaved in an identical manner to the normal antibodies with regard to fine antigen-binding reactivity (a Fab fragment feature) and protein A binding capacity (a feature requiring integrity at the C_H2 and C_H3 domain-interaction regions in the constant region of the heavy chain (C_H)). However, they lost the ability to activate complement, to bind to Fc receptors on macrophages, and to induce antibody-dependent cellular cytotoxicity. Furthermore, antigen–antibody complexes produced from such carbohydrate-deficient antibodies failed to be eliminated rapidly from the circulation. We conclude that removal of carbohydrate chains from IgG molecules may have a profound and highly select impact on the biological activity to these antibodies.**

Carbohydrates have been indicated to be of significant importance in secretion of Ig molecules (1), recognition of polymeric antigens (2), and creation of IgG–IgG complexes (3). Likewise complement fixation, opsonic activity, and the binding to Fc receptors of rabbit IgG antibodies may require carbohydrate moieties (4, 5). We have taken advantage of the availability of monoclonal antibody-producing hybridoma cells to obtain homogeneous antibodies with defined specificity. As carbohydrates on IgG and IgM are asparagine-linked (6–8), tunicamycin (Tm) blocking of the dolichol-dependent, asparagine-linked glycosylation pathway (9) can be used *in vitro* to obtain, through biosynthesis, carbohydrate-deficient Ig molecules (1). Our comparison of monoclonal IgG2b antibodies with or without carbohydrate chains demonstrates that the absence of carbohydrates will lead to an inability of the antibodies to function in antibody-dependent cellular cytotoxicity (ADCC), to interact with Fc receptors on macrophages, to activate complement, and to be eliminated rapidly from the circulation only when complexed with antigens. On the other hand, no reduction in protein A binding capacity or in affinity of the antibodies was noted upon carbohydrate depletion. The implications of these findings will be discussed.

MATERIALS AND METHODS

Cells and Reagents. Mouse anti-trinitrophenyl (anti-TNP) antibody-producing hybridoma cells, GKH-1 (ORK IgG2b, Hy 1.2 IgG2a, Hy2.15Ag1 IgG1, and Sp6 IgM), were gifts from Georges Köhler (Basel Institute for Immunology). The murine macrophage cell line M1 was provided by Yasou Ichikawa (Kyoto University Chest Disease Research Institute). Cells were kept in Dulbecco's modified Eagle's medium (DME medium) containing 10% heat-inactivated fetal calf serum (GIBCO), 100 μg of streptomycin per ml, 100 units of penicillin per ml, and 10 mM Hepes. Peritoneal cells were obtained from untreated 4-mo-old CBA/H mice by lavage. Spleen cells were prepared by gently teasing the spleen. Sheep erythrocytes (SRBC) stored in Alsever's solution at 4°C were used.

Tm was donated by Akira Takatsuki (Tokyo University, Faculty of Agriculture). Bovine serum albumin fraction V (Sigma), Sephadex G-25 and G-200, Sepharose 4B, CNBr-activated Sepharose 4B, protein A-Sepharose CL-4B, and protein A from *Staphylococcus aureus* (Pharmacia); L-[4,5-^{3}H]leucine (136 Ci/mmol; 1 Ci = 37 GBq), D-[U-^{14}C]glucosamine (277 mCi/mmol), $Na_2{}^{51}CrO_4$ (150–250 μCi/μg of Cr), and $Na^{125}I$, carrier free (Radiochemical Centre), were used.

Biosynthetic Radiolabeling of Ig and TNP Binding Assay. Hybridoma cells were put in the labeling medium (F10 medium without L-leucine containing 15% fetal calf serum pretreated with TNP-coated SRBC, 4 mM of L-glutamine, 100 μg of streptomycin per ml, 100 units of penicillin per ml, and 10 mM Hepes) at a cell density of 1×10^6 cells per ml. In some experiments (see Fig. 1), hybridoma cells (0.2 ml) were cultured in microplates (Falcon 3040) with Tm for 1 hr. [^{3}H]Leucine and [^{14}C]glucosamine (1 μCi each) were then added to each sample. After 18 hr, the microplates were centrifuged for 30 min at 1,000 × *g* to collect the supernatant. In other experiments (see Table 1), hybridoma cells precultured with Tm in DME medium for 6 hr, followed by washing in Hanks' balanced salt solution, were cultured with [^{3}H]leucine and [^{14}C]glucosamine (5 μCi/ml each) in a tissue culture flask (Nunc) in the renewed presence of Tm for 18 hr. Culture supernatants were collected after centrifugation at 4,000 × *g* for 30 min and used as antibodies.

Incorporation of [^{3}H]leucine and [^{14}C]glucosamine into anti-TNP antibodies was analyzed by binding to TNP-SRBC prepared as described (10). Each sample (0.1 ml) was mixed with 4×10^7 cells of TNP-SRBC or SRBC in 0.1 ml of 0.1% gelatin/Veronal buffer containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.4 (GVB^{2+}) in a microtiter plate (Cooke, UK). After incubation for 1 hr at 4°C, TNP-SRBC or SRBC were washed in GVB^{2+}, dissolved in 0.2 ml of 1% $NaDodSO_4$ solution, and transferred to 2 ml of a 2:1 (vol/vol) toluene/Triton X-100-based scintillation solution. Ig specifically bound to TNP was determined by subtracting the radioactivity bound to SRBC, which was always <10% of that of TNP-SRBC. Furthermore, adding TNP-bovine serum albumin (TNP-albumin) in excess did inhibit >95% of the binding of Ig to TNP-SRBC (data not included). The antibody level of each sample also was measured by hemagglutination (11) of TNP-SRBC.

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Abbreviations: Tm, tunicamycin; SRBC, sheep erythrocytes; TNP, trinitrophenyl; ADCC, antibody-dependent cellular cytotoxicity; TNP-albumin, TNP-bovine serum albumin; C_H, constant region of heavy chain.

Cytotoxicity Assays. Cytotoxicity was determined by release of ^{51}Cr from TNP-SRBC targets (12). In complement-mediated lysis, 10^5 targets were incubated with antibodies in 0.2 ml of GVB^{2+} containing guinea pig complement at 37°C for 1 hr. In ADCC 10^5 M1 cells or 10^7 spleen cells as effectors were incubated with 10^5 targets in 0.2 ml of DME medium with antibodies at 37°C for 15 hr. The percentage of chromium released was calculated from the formula: [(ER − CR)/(MR − CR)] × 100, where ER is experimental release, CR is control release in the presence of complement or effector cells without antibodies, and MR is maximum release obtained by the incubation with 0.05% of Nonidet P-40 solution.

IG-Coated TNP-SRBC and Fc Rosettes. Ig-coated TNP-SRBC were prepared (13) by using control or carbohydrate-depleted IgG2b antibodies in the same subhemagglutination titer. TNP-SRBC treated with labeling medium only were used as control TNP-SRBC. To assay Fc rosettes (14), mouse peritoneal cells or M1 cells were used.

Purification of IgG2b and ^{125}I-Labeling of Proteins. IgG2b antibodies in the culture supernatant of GORK hybridoma cells cultured in the presence or absence of Tm were purified by using protein A-Sepharose CL-4B (15). Some IgG2b antibodies were further purified on TNP-albumin-Sepharose 4B (16, 17).

Control and carbohydrate-depleted IgG2b purified by protein A, followed by TNP-albumin immunoabsorbent elution (100 μg each), were iodinated with 1 mCi of $Na^{125}I$ (18). TNP-albumin and protein A were likewise iodinated.

Polyacrylamide Gel Electrophoresis. Internally radiolabeled, protein A-bound, and eluted IgG2b was analyzed by 7% $NaDodSO_4$/polyacrylamide gel electrophoresis as described by Weber and Osborn (19), after reduction by heating in an 80°C water bath for 5 min in the loading buffer (1% $NaDodSO_4$/0.01 M phosphate buffer, pH 7.0/5% 2-mercaptoethanol). After electrophoresis the disk gels were frozen and sliced into 1-mm slices. The radioactivity of [^3H]leucine and [^{14}C]glucosamine in each slice was determined after elution into 0.3 ml of 1% $NaDodSO_4$ by incubation on a shaker at 37°C overnight and transfer to a scintillation solution.

Preparation of Immunocomplexes and Elimination Studies *in Vivo*. ^{125}I-Labeled TNP-albumin (6 μg) was mixed with an excess of protein A-purified, control or carbohydrate-depleted IgG2b (1 mg) in 1 ml of 0.14 M NaCl/0.01 M sodium phosphate, pH 7.2 (P_i/NaCl) at 37°C for 30 min. Each mixture was gel-filtered on a 10-ml Sephadex G-200 column. The excluded pool (>97% of total ^{125}I-labeled TNP-albumin) was analyzed in a 10-ml Sepharose 4B column. The general size distribution of either type of immunocomplex was found to be quite similar, admittedly with still some 50% of immunocomplexes being in the excluded fraction. They were used as control or carbohydrate-depleted immunocomplexes.

Elimination studies of immunocomplexes from the circulation were performed on 4-mo-old CBA/H mice as described (20). ^{125}I cpm in serum samples (0.1 ml each) and in their precipitates from 50% ammonium sulfate were determined.

RESULTS

Depletion of Carbohydrate Chains on Ig Molecules. The TNP-specific hybridoma cells were screened for sensitivity to Tm-induced inhibition of secretion in relation to carbohydrate depletion. The IgG2b-producing GORK line allowed secretion of Ig molecules to a significant extent even when carbohydrate depletion approached 100% (Fig. 1). In contrast, the IgM-producing hybridoma was more sensitive to inhibition of secretion than to removal of carbohydrate moieties. IgG2a and IgG1 hybridoma cells behaved like the IgM hybridoma. Thus, the GORK

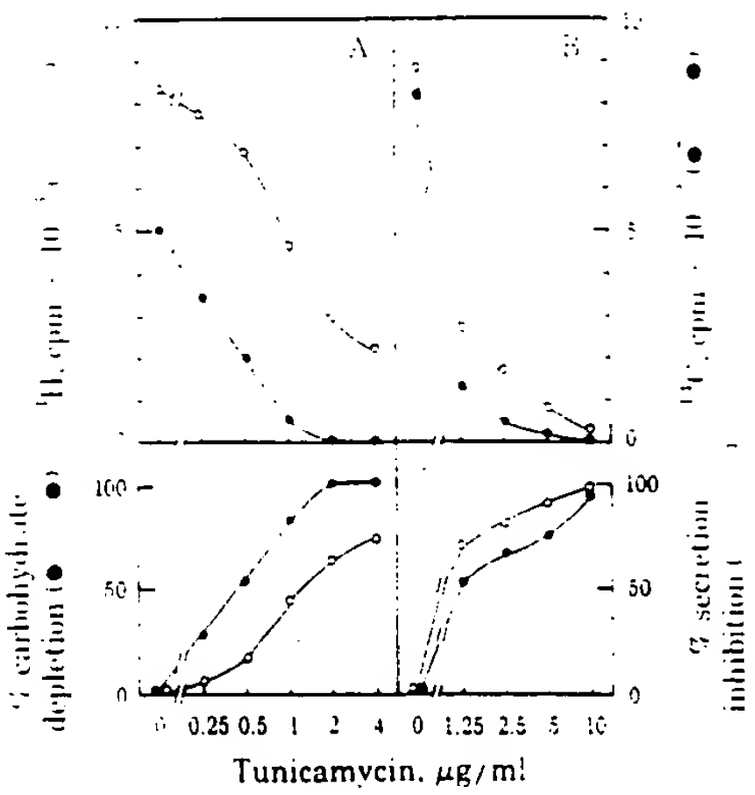

FIG. 1. [^3H]Leucine and [^{14}C]glucosamine incorporated into anti-TNP antibodies. (*A*) GORK IgG2b. (*B*) Sp6 IgM. Cells were cultured with the indicated concentration of Tm for 1 hr prior to biosynthetically labeling with [^3H]leucine and [^{14}C]glucosamine. After 18 hr, radioactivity of secreted antibodies was analyzed by the TNP binding assay. Percentages of secretion-inhibition and carbohydrate-depletion were calculated from the following formulas, respectively:

$$\left(1 - \frac{^3H \text{ cpm with Tm}}{^3H \text{ cpm without Tm}}\right) \times 100;$$

$$\left(1 - \frac{^{14}C \text{ cpm}/^3H \text{ cpm with Tm}}{^{14}C \text{ cpm}/^3H \text{ cpm without Tm}}\right) \times 100.$$

Each point represents the mean of duplicate samples.

cell line was chosen for further studies.

We next analyzed the time required after Tm administration to allow the intracytoplasmic pool of antibody molecules to turn over. When GORK cells preincubated with [^3H]leucine were cultured, >99% of the intracytoplasmic pool of IgG2b antibodies (^3H-positive) was secreted during the first 6 hr, regardless of the presence of Tm. Accordingly we chose to preincubate GORK cells with Tm for 6 hr, followed by washing before radiolabeling, as shown in Table 1. IgG2b antibodies obtained from such Tm-treated GORK cells were dramatically reduced in [^{14}C]glucosamine content while retaining normal hemagglutination titers when compared at the same protein ([^3H]leucine)

Table 1. Anti-TNP monoclonal IgG2b antibodies depleted of carbohydrate contents in the culture supernatant of GORK hybridoma cells

Batch	Tm, μg/ml	Incorporation into antibodies bound to TNP-SRBC, cpm: [^{14}C]Glucosamine	Incorporation into antibodies bound to TNP-SRBC, cpm: [^3H]Leucine	% carbohydrate depletion*	Hemagglutination of TNP-SRBC, dilution titer
A	0	965 ± 130†	7,780 ± 220		3×2^5
	1.5	50 ± 10	3,055 ± 185	87.1	3×2^4
B‡	0	3,035 ± 260	4,405 ± 305		3×2^5
	1.0	148 ± 9	1,740 ± 105	87.7	3×2^4
C	0	362 ± 50	7,708 ± 194		3×2^5
	1.0	53 ± 16	4,700 ± 78	80.9	3×2^4

* Calculated according to the formula shown in Fig. 1.
† The mean cpm of triplicate samples ± SD.
‡ DME medium was used instead of F10 medium.

concentration. Moreover, by using $NaDodSO_4$-polyacrylamide gel electrophoresis, protein A-bound and eluted Ig molecules (batch C, Table 1) were shown to be highly depleted of carbohydrate contents normally linked to the heavy chains of IgG2b molecules (not shown). In addition, no significant amount of split peptide fragments of carbohydrate-depleted IgG2b molecules was observed.

Impact of Carbohydrate Deficiency in IgG2b Antibodies. *Failure to influence fine antigen-binding capacity or protein A binding ability.* The Farr precipitation assay will detect differences in avidity (21). Absence of carbohydrates had no detectable effect on the capacity of IgG2b antibodies to react with TNP (Fig. 2A). Protein A from *Staphylococcus aureus* has the select ability to react with a region involving the C_H2 and C_H3 domains of the heavy chain constant regions (C_H) of most mammalian IgG classes (23, 24). Normal and carbohydrate-deficient IgG2b antibodies displayed identical protein A binding capacity (Fig. 2*B*). C_H2 or C_H3 domains do not alone bind to protein A (25). The interaction requires an intact C_H2-C_H3 interface (24). Thus, we can conclude that absence of carbohydrates has no general effect on conformation of the Fc region of IgG2b antibodies as measured by protein A.

Reduction in Fc receptor binding ADCC ability. Fc receptors on mouse macrophages recognize sites present at the C_H2 domains of mouse IgG2b molecules (14). Normal and carbohydrate-deficient IgG2b anti-TNP antibodies were used for coating TNP-SRBC and subsequent testing for rosette formation with peritoneal macrophages or the macrophage cell line M1 (26). Carbohydrate-deficient antibodies largely failed to induce such rosette formation (percentage reduction, 91% for M1 cells and 90% for peritoneal macrophages). Likewise, when the ability of ^{125}I-labeled TNP-albumin–anti-TNP immunocomplexes to bind to these cells was analyzed, no significant binding was noted in the complexes made up from carbohydrate-deficient antibodies (Fig. 3*A*). No differences in the formation of both types of antibodies were noted when measured in another assay, the Farr system (Fig. 3*B*). Furthermore, in ADCC with either M1 or whole spleen cells as effector cells (Fig. 4), antibodies lacking carbohydrates were virtually inactive as inducing agents ($^1/_{16}$ to $^1/_8$ as efficient as the corresponding normal antibody molecules). Thus, the data obtained would indicate that the carbohydrate chains present in the IgG2b C_H2 domains are fundamental in creating a proper recognition between IgG and the macrophage Fc receptor.

Reduction in complement activation capacity. Our studies had shown that binding to TNP-coated erythrocytes was identical for normal and carbohydrate-deficient antibodies (Table 1). The ability of the latter to lyse TNP-SRBC was, however, significantly impaired in complement-mediated lysis (Fig. 5; ≈75% reduction). To exclude the possibility that natural antibodies present in guinea pig serum against murine IgG2b carbohydrates may cause the observed differences, we also used guinea-pig complement pretreated with a normal GORK IgG2b immunosorbent. The results demonstrated, however, an even greater reduction in complement-activating capacity of the carbohydrate-deficient antibodies (Fig. 5). The binding site for the C1 complement factor is localized to the C_H2 domain of IgG (27). The reduction in complement-inducing ability was in the same order of magnitude as the carbohydrate depletion (see

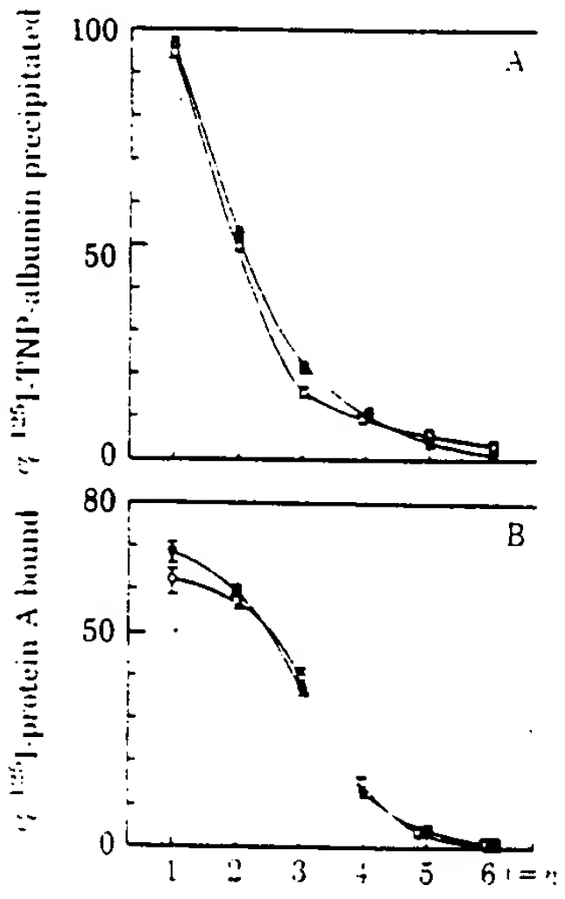

FIG. 2. Antigen-binding capacity of IgG2b antibodies (*A*) and protein A binding capacity (*B*). The antibodies (not purified; batch A of Table 1)—control (○) and carbohydrate-depleted (●)—were used in the same hemagglutination in both experiments. (*A*) Based on the Farr technique (22), ^{125}I-labeled TNP-albumin (^{125}I-TNP-albumin) (0.14 μg; × 10^4 cpm) was incubated with the antibodies serially diluted in 0.5 ml of the labeling medium at 4°C overnight, and then the 50% ammonium sulfate-precipitable radioactivity of ^{125}I-TNP-albumin was assayed. Ammonium sulfate precipitation of ^{125}I-TNP-albumin alone in the labeling medium did not exceed 5% of trichloroacetic acid-precipitable radioactivity. (*B*) ^{125}I-Labeled protein A (^{125}I-protein A) (0.1 μg; × 10^5 cpm) was incubated with IgG2b-coated TNP-SRBC or noncoated TNP-SRBC serially diluted in 50 μl of 1% ovalbumin/P_i/NaCl (initial cell number, 5×10^6). After incubation at 4°C for 1 hr, each sample was washed in 1% ovalbumin/P_i/NaCl, and then the radioactivity specifically bound to IgG2b-coated TNP-SRBC was determined by subtracting the amount of ^{125}I-protein A bound to noncoated TNP-SRBC. Each point represents the mean of triplicate samples ± SD, expressed as the percentage of 10% trichloroacetic acid-precipitable radioactivity of administered ^{125}I-TNP-albumin or ^{125}I-protein A.

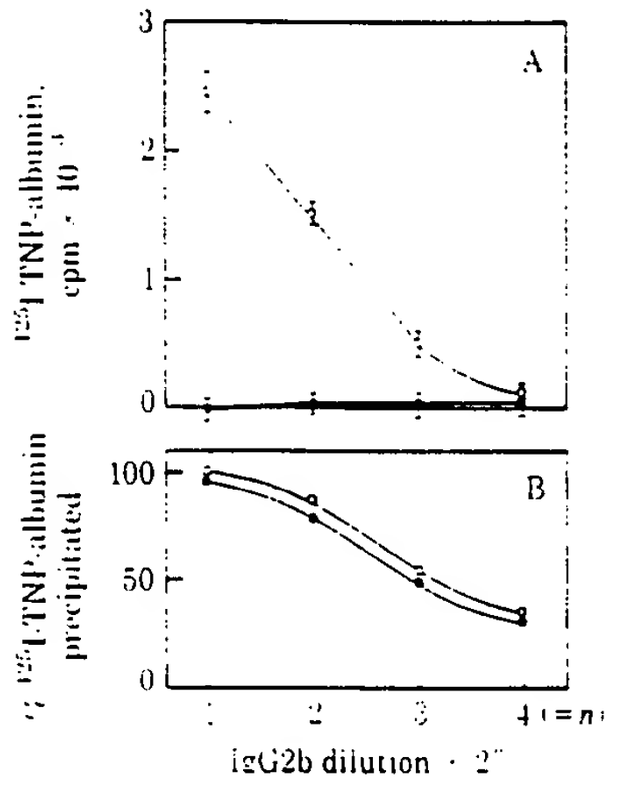

FIG. 3. Immunocomplex binding of M1 cells. Immunocomplexes were prepared in the mixture of ^{125}I-labeled TNP-albumin (^{125}I-TNP-albumin) (0.3 μg; 1 × 10^5 cpm) and 1 ml of the antibodies (not purified; batch A of Table 1)—control (○) or carbohydrate-depleted (●)—which had been adjusted to the same hemagglutination and serially diluted with the labeling medium. (*A*) M1 cells (10^5) were incubated in 0.1 ml of the mixture of immunocomplexes at 4°C for 1 hr. After the cells were washed, the radioactivity of ^{125}I-TNP-albumin bound to M1 cells was assayed. Each point represents the mean cpm ± SD from triplicate samples. (*B*) The relative amount of immunocomplexes in the mixture was tested by the Farr technique (see Fig. 2*A*). Each point represents the mean from duplicate samples.

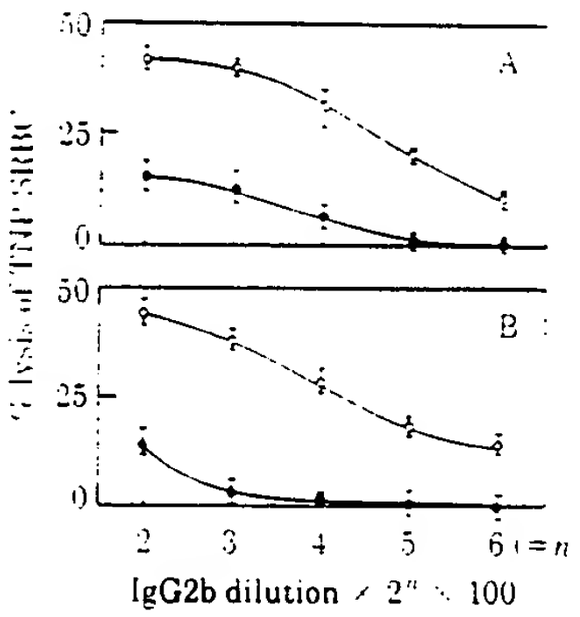

FIG. 4. ADCC of M1 cells (*A*) and spleen cells (*B*) against ^{51}Cr-labeled TNP-SRBC. IgG2b antibodies—control (○) and carbohydrate-depleted (●)—which had been purified from batch B of Table 1 by a protein A column, were used in the same hemagglutination. Each point represents the mean of triplicate samples ± SD. Control release of ^{51}Cr-labeled TNP-SRBC was 21% of the incorporated count; maximum release was 96%.

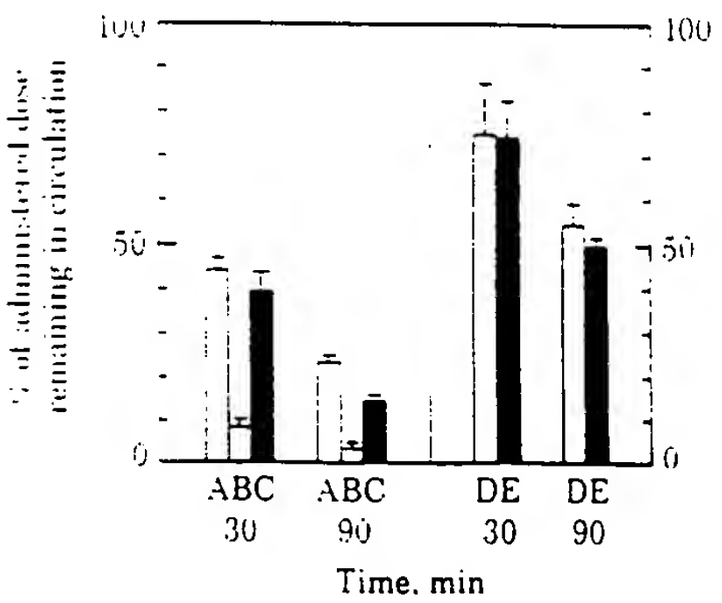

FIG. 6. Elimination of IgG2b complexes (*Left*) and IgG2b antibodies alone (*Right*). Lanes: A, antigen alone [^{125}I-labeled TNP-albumin (^{125}I-TNP-albumin)]; B, control IgG2b complexes (^{125}I-TNP-albumin + control IgG2b); C, carbohydrate-depleted IgG2b complexes (^{125}I-TNP-albumin + carbohydrate-depleted IgG2b); D, ^{125}I-labeled control IgG2b; E, ^{125}I-labeled carbohydrate-depleted IgG2b. Each point represents the mean ± SD from at least four mice expressed as the percentage of the administered dose of radioactivity remaining in circulation 30 min or 90 min after injection. Values for lanes B, C, D, and E were calculated from 50% ammonium sulfate-precipitable radioactivity.

ible 1). Thus, absence of the carbohydrate chains normally localized to C_H2 domains may inactivate the C1 site as to its reducing/binding ability.

Failure of immunocomplexes to display a rapid elimination in vivo. We next studied the capacity of carbohydrate-deficient antibodies *in vivo* to cause elimination of immunocomplexes from the circulation. ^{125}I-Labeled TNP-albumin was used to create complexes with either normal or carbohydrate-deficient antibody molecules. The rate of elimination of such immunocomplexes from the circulation did differ dramatically, at least initially; whereas normal antibody–antigen complexes were rapidly eliminated, carbohydrate-deficient antibody–antigen complexes were eliminated at the same rate as antigen alone (Fig. 6 *Left*). This difference was further emphasized by the finding that the amount of antigen still in combination with antibody in serum after 30 and 90 min was only 67.5% and 35.8% for the normal antibody–antigen complexes in contrast to 93.0% and 68.8% for the carbohydrate-deficient complexes (analyzed as radioactivity precipitable in 50% ammonium sulfate solution). On the other hand, carbohydrate-deficient antibodies alone were almost eliminated in the same manner as control Ig molecules (Fig. 6 *Right*).

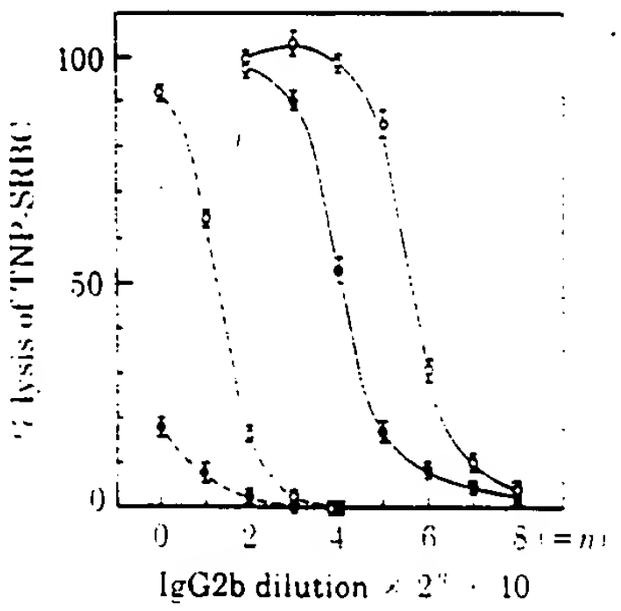

FIG. 5. Complement-mediated cytotoxicity of IgG2b antibodies against ^{51}Cr-labeled TNP-SRBC. Both antibodies (not purified; batch 1 of Table 1)—control (○) and carbohydrate-depleted (●)—were used in the same hemagglutination. The antibodies in serial dilutions were incubated with ^{51}Cr-labeled TNP-SRBC in the presence of guinea pig complement, which had been absorbed with TNP-SRBC (——) or control IgG2b-coated TNP-SRBC (----) in P_i/NaCl at 4°C. Each point represents the mean of triplicate samples ± SD. Control release of ^{51}Cr-labeled TNP-SRBC was 9% of the incorporated count; maximum release was 95%.

DISCUSSION

We have attempted to analyze the role of carbohydrate chains on monoclonal antibody molecules in their biological functions. Tm, a select inhibitor of dolichol-dependent glycosylation (9, 29), in combination with an anti-TNP reactive hybridoma cell line, GORK, allowed production and secretion of carbohydrate-deficient IgG2b molecules in useful amounts. Absence of carbohydrate chains in the C_H2 domains of IgG2b antibodies (29) induced by this procedure had drastic and select consequences as to antibody effector functions. No impact was noted with regard to fine antigen-binding specificity or ability to react with protein A from *Staphylococcus aureus*. However, carbohydrate removal did reduce, close to completely, some other biological functions of the Ig molecules; namely, ADCC, binding to Fc receptors on macrophages, complement activation, and rapid elimination of antibody–antigen complexes from the circulation.

The C_H2 domains of IgG2b are known to be essential for efficient binding to the corresponding Fc receptors on macrophages (14). The binding site for C1q is also localized to the C_H2 domain of IgG (27). Yet several sets of results indicate that additional conformational features of Ig molecules contribute to Fc receptor binding and ability to activate complement. For instance, variations in C_H1 or the hinge region, or both, may completely eliminate Fc receptor binding capacity (30, 31) and also may affect seriously complement activating capacity (31, 32). On the other hand, the carbohydrate chains in the corresponding human IgG1 molecules not only cover a hydrophobic patch in C_H2 domains (33) but extend to involve regions between C_H1 and C_H2 (34). Thus, it is possible that allosteric changes of IgG molecules because of binding to "rigid" antigens may lead to a carbohydrate chain-dependent change in C_H2. The carbohydrate chains in Ig molecules in aggregates or immunocomplexes are also more flexible (35) and accessible (36) than in the native antibody molecule. It is possible that such carbohydrate chains directly participate in the reaction with the Fc receptors or C1q or both. Alternatively, their increased flex-

ility may merely reflect changes in the polypeptide structure C_H2 domains, making these reactive with Fc receptors or complement factors.

The region between C_H2 and C_H3 recognized by protein A from staphylococci (23–25) is unchanged in the carbohydrate-lacking antibodies, excluding earlier claims that the Fc receptors and protein A react with the very same steric configurations on IgG molecules (37). Studies on hybrid molecules of IgG2b and IgG2a have indicated that the ability of C_H2 domains to exert Fc receptor binding or complement binding, or both, may be indirectly impaired by amino acid changes outside C_H2 (30). However, no studies as to the possible role of carbohydrates were performed in these experiments. The present studies and those involving actual modifications of the peptide parts of Ig molecules suggest the possibility of obtaining antibodies devoid of select biological functions. Such reagents not only may have potential therapeutical possibilities but also should help to elucidate basic underlying mechanisms of the biological function of Fc regions of IgG antibodies.

We are grateful for the help of Dr. M. Zöller in culturing hybridoma cells used in these studies and for the technical advice of Dr. M. Gidlund. We thank Drs. M. Kyogoku (Tohoku University, Sendai), A. Kosata (Tokyo University, Tokyo), and R. Huber (Max-Planck-Institute, Martinstried) for helpful suggestions and encouragement. The advice of Dr. C. Dahl in the preparation of this manuscript and the secretarial assistance of Mrs. G. Arvidson are also highly appreciated. This work was supported by a grant from the Swedish Cancer Society.

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Exhibit B-3

Ann. Rev. Biochem. 1988. 57:785–838

GLYCOBIOLOGY

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CONTENTS

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PERSPECTIVES AND SUMMARY

It has become evident that a reevaluation of the biological role of oligosaccharides is necessary. Oligosaccharides exist mainly in covalent association with proteins or lipids, consequently both intramolecular and intermolecular functions are simultaneously influenced by the carbohydrate. Changes in protein stability, rate of proteolysis, thermal stability, solubility, etc that occur when glycosylation variants are generated experimentally support the idea that intramolecular interactions involving both carbohydrate and polypeptide define the physical properties of a glycoprotein. Paradoxically, dramatic changes in the structures of the oligosaccharide moieties of glycoconjugates often are associated with oncogenic and ontogenic events without affecting cell viability. The structural and functional roles of oligosaccharide moieties of proteins are interdependent and controlled variation in the structures is necessary. At any developmental stage cells may have solved the biosynthetic problem of controlled variation by making not just one glycoprotein (i.e. protein with a single oligosaccharide at each site) but by *coding* for large repertoires of a protein, each variant with different covalently attached oligosaccharides. This pool of protein *glycoforms* would contain distinct members, each of which may have a unique spectrum of biological activities while maintaining the intramolecular characteristics necessary for structural integrity. Hence, a glycoprotein has composite activity, which a cell can control by varying the relative incidence of each glycoform. Displaying the entire spectrum of oligosaccharides simultaneously (i.e. microheterogeneity) may be necessary to avoid immunogenicity at later developmental stages.

A more flexible mechanism that allows for the interdependence of the structural and functional roles of oligosaccharides can be envisaged if the polypeptide directs its own glycosylation. The biosynthesis of oligosaccharides while covalently attached to folded peptide domains appears to be controlled in part by polypeptide-oligosaccharide interactions involving stabilization of oligosaccharide conformers by peptide. Recognition of, or failure to interact with, specific oligosaccharide conformers (in addition to steric restraints) by processing enzymes, continues or diverges the biosynthetic pathway. Control of the expression of glycosidases and transferases by the cell gives rise to cell-type–specific glycosylation patterns. Clearly, the separation and isolation of individual glycoforms should provide researchers with natural variants to probe the functional properties of the oligosaccharide moieties of glycoproteins.

In this review we hope to place into perspective those experimental findings that are directing the current theories concerning the role of N-linked oligosaccharides in mediating protein-specific biological activity. The role of N-linked oligosaccharides in modulating the physicochemical properties of a

glycoprotein will not be discussed. Several other reviews have appeared dealing with various aspects of glycoproteins or oligosaccharides: biosynthesis (1, 1a), oligosaccharide structure (2), oligosaccharides as antigenic determinants on glycoproteins (3, 4), glycosylation mutants (5), intracellular targeting (6), physicochemical properties (7), and solution conformation (8). In addition, we note the recent recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature concerning the nomenclature of glycoproteins, glycopeptides, and peptidoglycans (9).

GLYCOFORMS

Definition

Characteristics of polypeptide N-glycosylation include the following [for a discussion see (2)]: (*a*) different glycoproteins from the same cell may contain different oligosaccharide structures; (*b*) an individual polypeptide usually carries several different structures; (*c*) many structures are found at the same glycosylation site (commonly referred to as site heterogeneity); (*d*) the pattern of oligosaccharide heterogeneity at a single glycosylation site during a constant physiological condition is reproducible and not random; and (*e*) the cell-type plays a role in oligosaccharide processing. This form of posttranslational modification of a polypeptide therefore serves to create discrete subsets (*glycoforms*) of a glycoprotein that have different physical and biochemical properties that may lead to functional diversity. Where there is only one glycosylation site, the number of glycoforms is simply the number of different oligosaccharide structures at that site. The number of possible glycoforms increases dramatically as either the number of glycosylation sites or the heterogeneity at each site increases. In short, glycosylation of a polypeptide creates a set of glycoforms all of which share an identical polypeptide but differ with respect to glycosylation (i.e. the same polypeptides with oligosaccharides that differ either in sequence or in disposition). The relative sizes (Figure 1) of the different oligosaccharides may also alter considerably the three-dimensional space occupied by such glycoforms.

A recent study of rat Thy-1 illustrates the need to consider glycoforms in defining a given polypeptide (10). This protein is the smallest member of the immunoglobulin superfamily (11), and in rats and mice is a major cell-surface glycoprotein of thymocytes and brain. The Thy-1 polypeptide has 111 amino acids, is attached to the membrane by a glycosyl-phosphatidylinositol anchor (12, 13), and is N-glycosylated at three sites (Asn-23, -74, and -98 in rat). Figure 2 demonstrates the relative sizes of the polypeptide and oligosaccharide parts of Thy-1. A significant contribution to the surface area of the molecule is made by the glycan moieties. Indeed, when the Thy-1 molecule is bound by its anchor to the membrane, the polypeptide appears to "float"

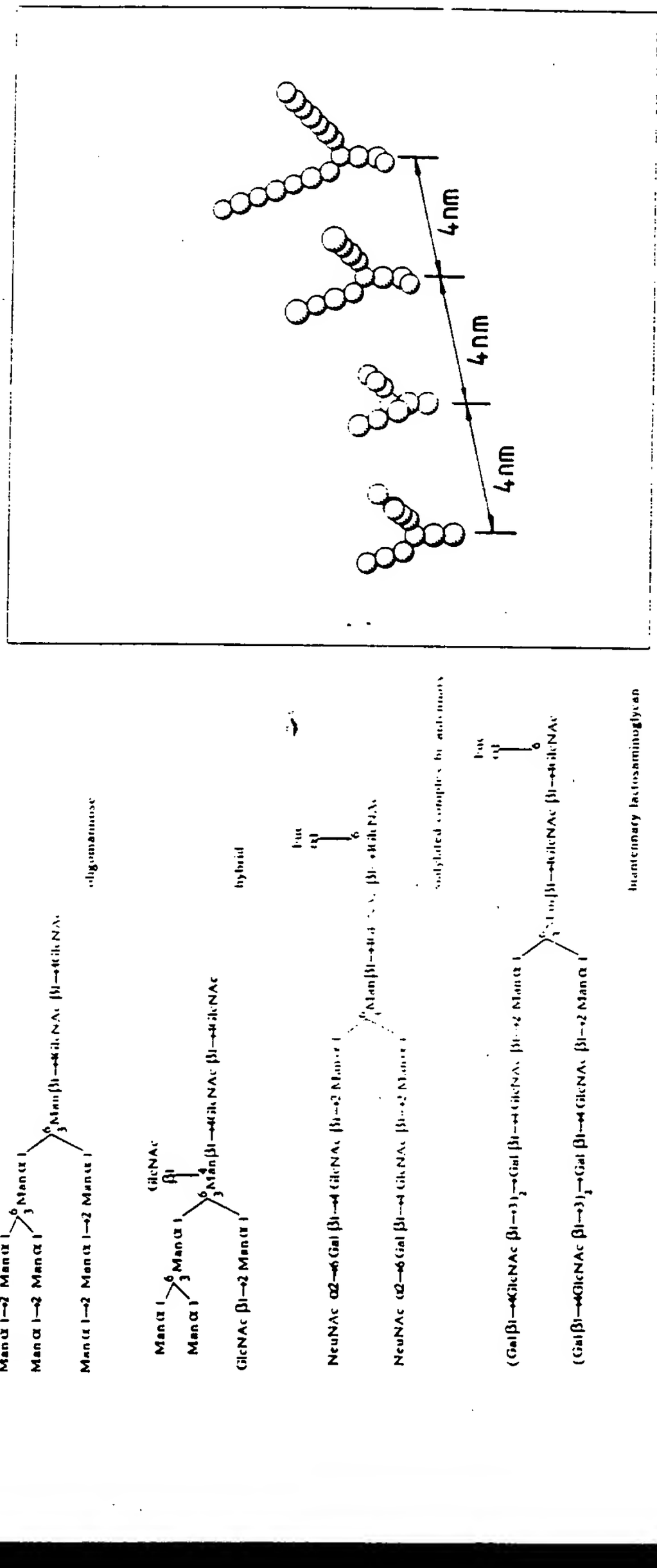


Figure 1. Diagram to show the relative sizes of four representative oligosaccharide side-chains and their structural configurations. Reading from left to right: oligomannose, hybrid, sialylated biantennary, and biantennary lactosaminoglycan.

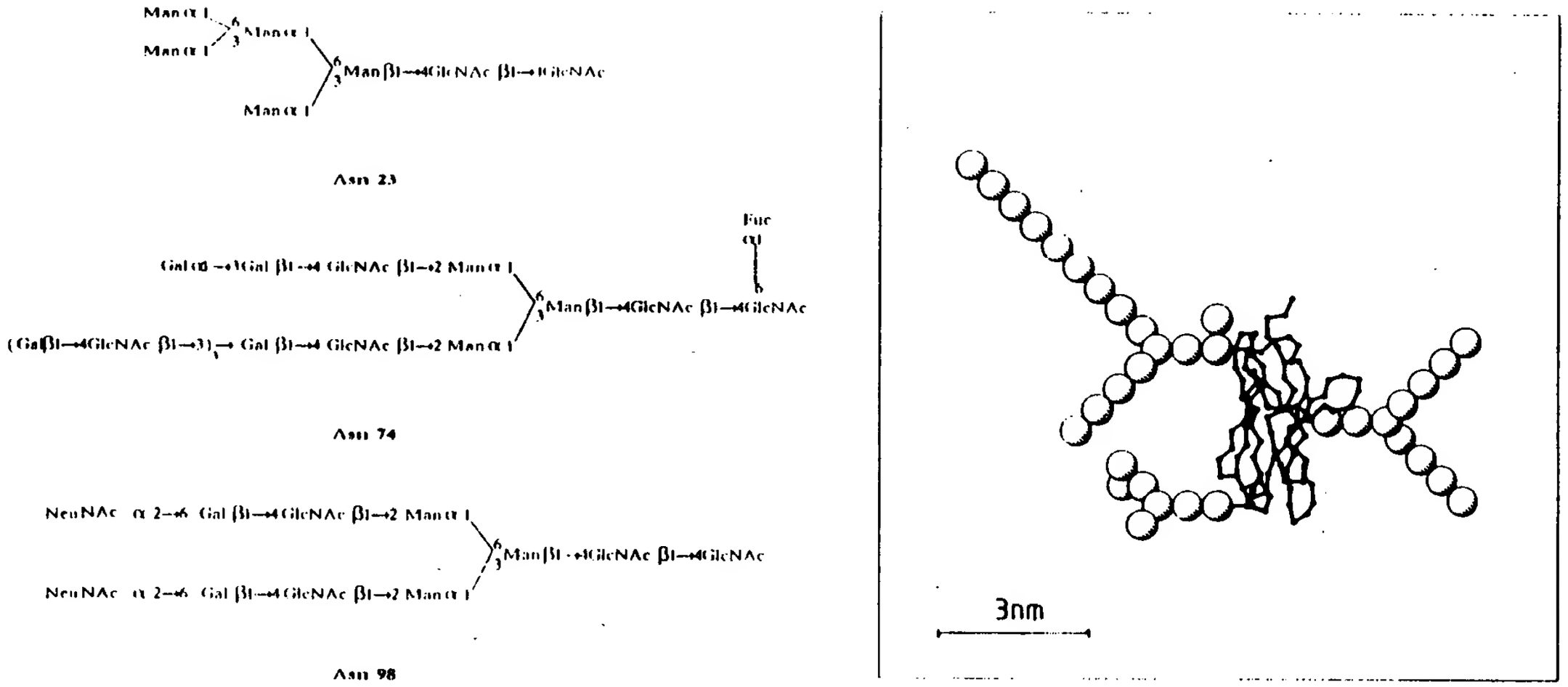


Figure 2. The Thy-1 molecule from rat thymocytes. This structure was generated by using the α-carbon coordinates from the immunoglobulin fold struture of the V_L domain of Fab New from the Protein Data Bank, which on the basis of sequence homology closely resembles Thy-1.1 (79). The oligosaccharides that occur at positions 23, 74, and 98 on Thy-1 are attached to the V_L framework at the appropriate places in Fab New [i.e. Ser 27, Gly 77, and Gly 101, numbering as in Kabat et al (290)], on the basis of defining the β-strands of the putative fold. The three oligosaccharides shown are representative of the known structures at the three sites respectively (10), and are in extended conformations perpendicular to the protein surface. Each sugar ring is represented by a sphere of 0.608 nm in diameter.

within an oligosaccharide shell (Figure 3). The solubilization of Thy-1 by release from its lipid tail (e.g. by PI-PLC, see 13a) would effectively expose a nascent fourth glycan moiety.

A set of "composite" glycoforms of rat Thy-1 based largely on a determination of the class of oligosaccharide at each site is shown in Figure 4. Any actual glycoform would be derived from a particular one of these composite forms by taking into account the microheterogeneity of the structures within each class. Comparing the set of glycoforms constituting rat brain Thy-1 to that constituting rat thymocyte Thy-1 shows that in the rat, brain Thy-1 and thymocyte Thy-1 do not have any glycoforms in common. Obviously, the two tissues have quite different glycosylation capacities (i.e. *glycotypes*—see section on SPECIES-SPECIFIC AND TISSUE-SPECIFIC N-GLYCOSYLATION). A complete definition of the Thy-1 molecule must therefore include a set of glycoforms and by this definition rat brain and thymocyte Thy-1 are different molecules.

Biosynthesis

Recent data suggest that oligosaccharide biosynthesis at a given glycosylation site is not independent of the biosynthetic events occurring at other glycosylation sites, thus giving rise to only certain allowed glycoforms. Such an interaction would be necessary if the oligosaccharides of cell-surface glycoproteins are recognized in clusters and not individually (10), and this could

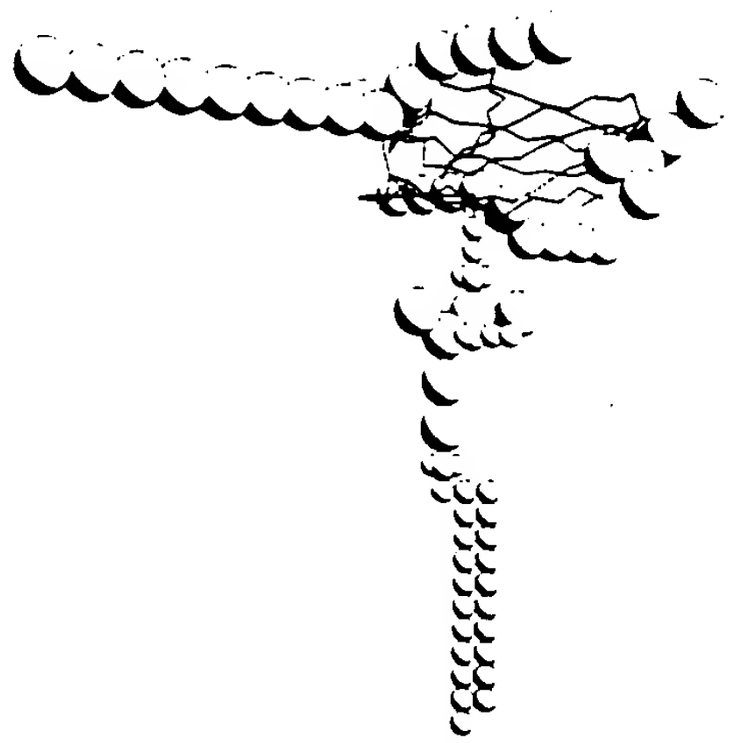


Figure 3. Schematic representation of Thy-1 with a glycophospholipid membrane anchor. The molecule is built up from that shown in Figure 2 by first making the disulfide bridge involving the terminal cystine and then by attaching a glycosylphosphatidylinositol (GPI) anchor (137). The glycan moiety in the anchor is linked via phosphate groups to the ethanolamine-protein and to the lipid. An additional ethanolamine is attached to a mannose residue of the glycan.

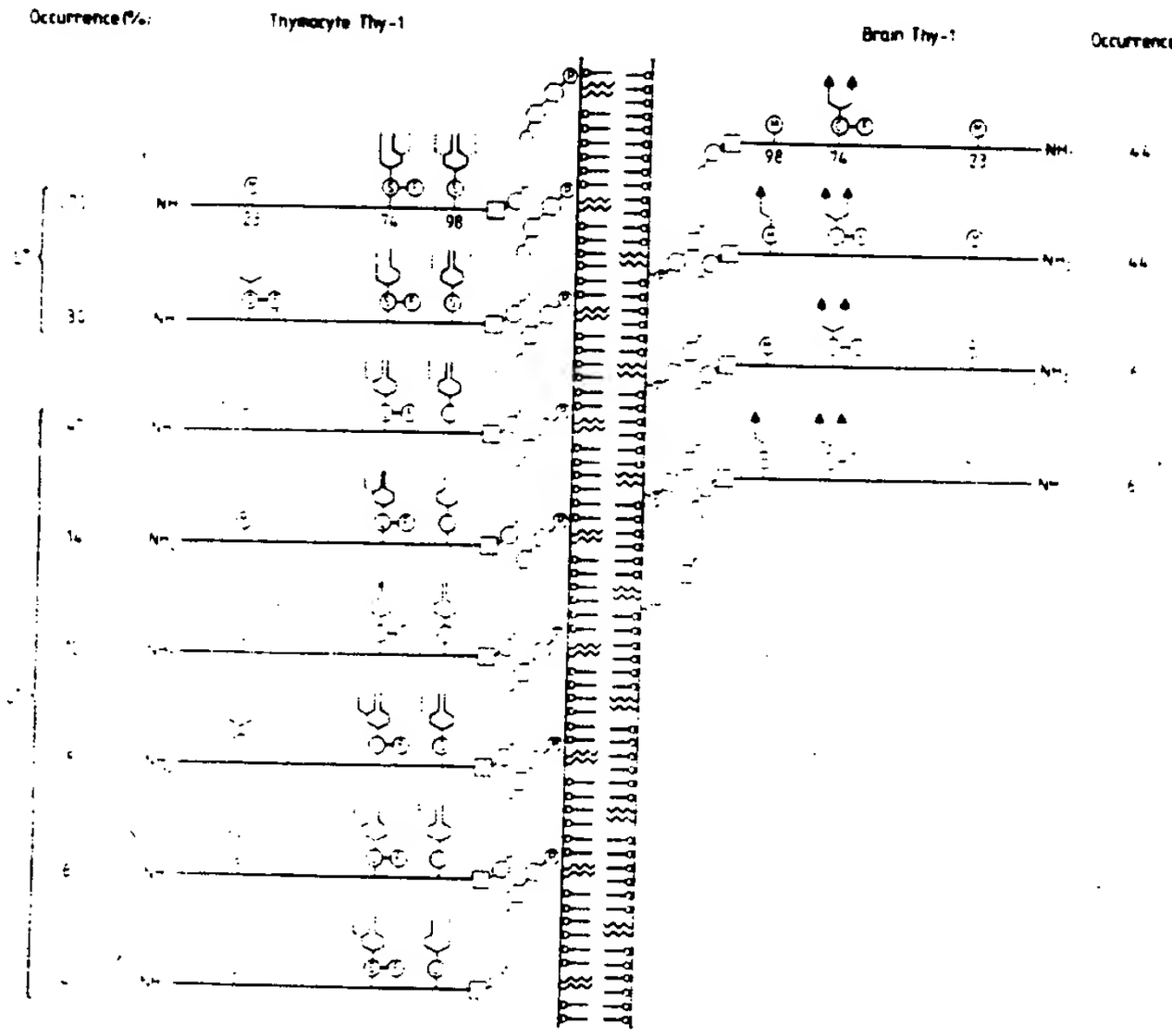


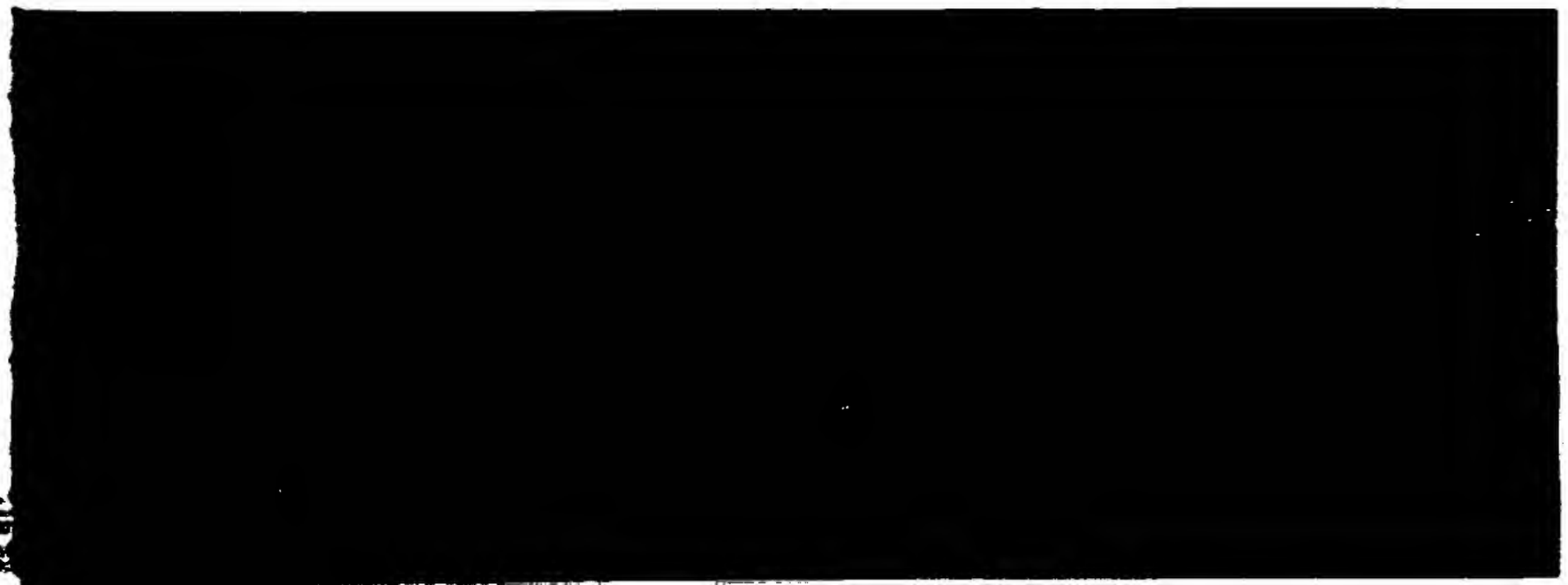
Figure 4. "Composite" glycoforms found in brain and Thymus Thy-1. The occurrence of each composite glycoform was calculated from the site distribution assuming that the glycosylation at each site was independent of the other sites. The Manα1→6Manβ→R branch is on the right, Symbols represent the type of structures present at each site—see Ref. 10 for details.

be one reason why the majority of glycoproteins contain multiple glycosylation sites. For example, thymocyte Thy-1 can be separated by lentil lectin–agarose chromatography into two fractions, termed Thy-1 L+ (lentil lectin–retarded) and Thy-1 L− (lentil lectin–unretarded). Lentil lectin has high affinity for structures with fucose linked at the C-6 position of the reducing terminal N-acetylglucosamine residue, GlcNAc-1 (i.e. "core" fucosylation). However, "core"-fucosylated triantennary structures containing outer chains linked at the C-2 and C-4 of Manα1→3(6) residues do not interact with the lectin (14–16). Comparing the N-glycosylation of thymocyte Thy-1 L+ and L− confirms the specificity of this lectin as determined using glycopeptides, and indicates that the fractionation by lentil lectin is mainly based on the nature of the triantennary structures at site 74 (10). Yet structures at the "passenger" sites (of Asn-23 and -98), which are not directly involved in lentil lectin binding also correlated with the separation (10). This indicates that N-glycosylation at one site may be influenced by events at different sites.

An analysis of the immunoglobulin G molecule provides clear evidence for direct interactions between N-linked oligosaccharides, with the result that only certain IgG glycoforms appear to be allowed (17). In IgG there is a conserved N-glycosylation sequon at Asn-297 in each of the C_H2 domains, and the two oligosaccharide units are in direct contact with each other, and form a bridge across the two domains (Figure 5). At least 30 complex-type biantennary oligosaccharides are associated with serum IgG (17, 18), but X-ray crystallographic (19), and oligosaccharide sequence studies (17, 18) have shown that there is a restriction in pairing of the two oligosaccharides across the domains. This leads to there being different monosaccharide sequences on the $\alpha(1\rightarrow3)$ arms of the two oligosaccharides in some Fc moieties. One of these arms must always be devoid of galactose, thereby exposing the $\beta(1\rightarrow2)$ GlcNAc residue, which then interacts with the Man-$\beta1(1\rightarrow4)$GlcNAc core segment of the opposing oligosaccharide chain. The $\alpha(1\rightarrow3)$ antenna of this latter chain can extend outward between the domains with no apparent steric restrictions on the primary sequence up to and including the terminal sialic acid residue. Despite having identical amino acid sequences, therefore, the two heavy chains of some IgG molecules carry N-linked oligosaccharides of different primary sequence. N-linked oligosaccharides are also attached to the Fab region of IgG (17, 20) with a frequency and location dependent on the occurrence of the Asn-Xaa-Ser(Thr) sequon in the hypervariable region (21, 22, 291). The occurrence and nature of Fab N-glycosylation will lead to further diversification of an IgG. Although the role of the Fab oligosaccharide is not known, there is increasing evidence that IgG forms containing Fab oligosaccharides may be preferentially involved in IgG self-association, aggregation, and cryoprecipitation (20, 23, 24) (see GLYCOSYLATION IN DISEASE section). Fractionation of other purified glycoproteins based on carbohydrate have been reported for α-1-antitrypsin (25), transferrin (26), and ovomucoid (27).

Control of Biological Activity through the Nature of Glycosylation

The glycosylation of a protein may contribute to and be an intrinsic part of its physiological activity. Any given glycoprotein that consists of different glycoforms will therefore have a "composite" activity, reflecting a weighted average of the activity and incidence of each glycoform. The set of glycoforms of a given polypeptide expressed by an organism is dependent on its physiological state. Examples include the controlled variations in the N-glycosylation of human serum transcortin and thyroxine-binding globulin (two hormone-binding serum glycoproteins) during pregnancy (28), changes in hepatic N-glycosylation of α_1-antitrypsin and α_1-acid glycoprotein



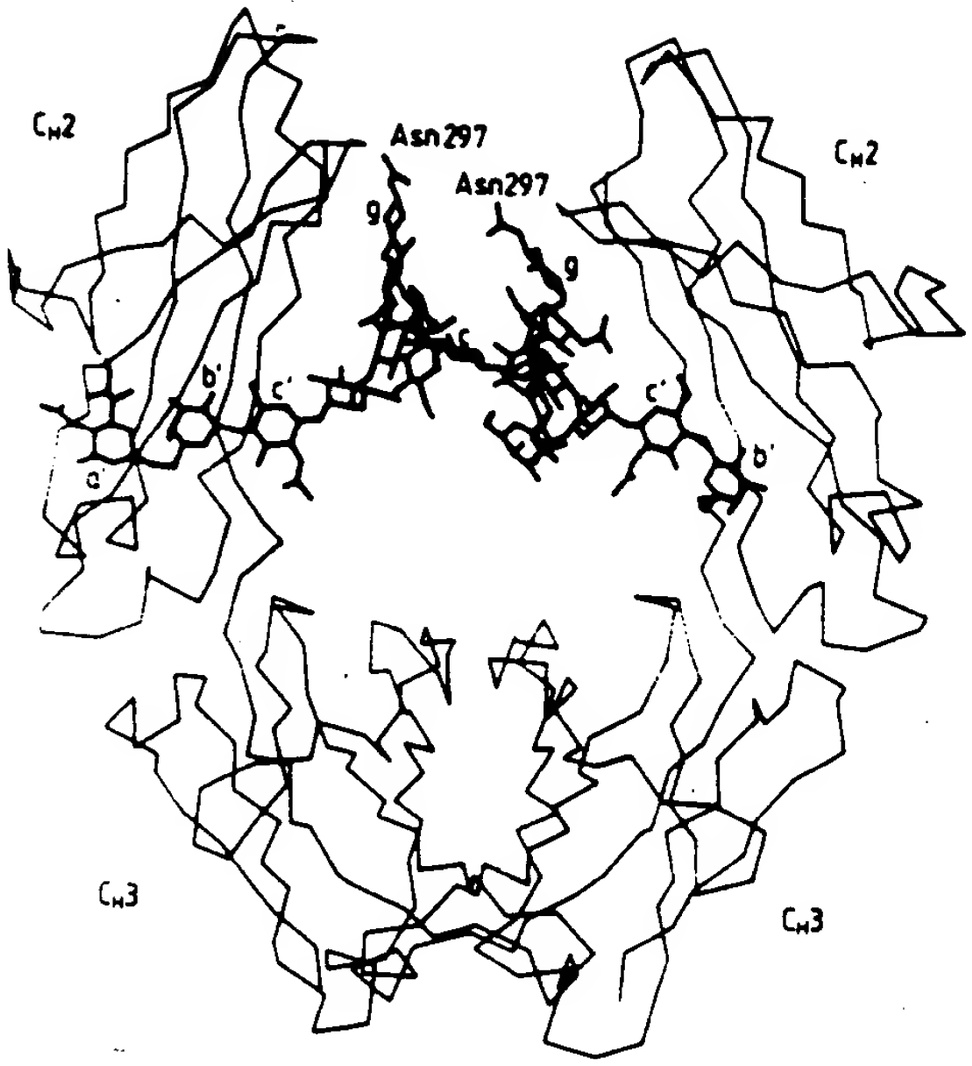


Figure 5 Refined structure at 2.8 Å of rabbit Fc fragment from the crystal data of Sutton & Phillips (19). The two carbohydrate chains, each attached at Asn-297, differ in conformation and may also differ in sequence and bridge the two C_H2 domains. The $\alpha(1\rightarrow3)$ arm of the chain (left side) is always devoid of galactose and interacts through its $\beta(1\rightarrow2)$-linked GlcNAc residue with the Man$\beta(1\rightarrow4)$GlcNAc segment of the opposing (right side) oligosaccharide chain. The $\alpha(1\rightarrow3)$ arm of the right chain extends outward between the domains with no apparent steric constraints on its length. A NeuNAc unit is shown on one $\alpha(1\rightarrow6)$ arm only (left). The electron density for this unit is weak and, experimentally, no disialylated oligosaccharide chains occur on the Fc. The letters a–g represent monosaccharide residues (see Ref. 18 for nomenclature).

during acute inflammation (29), and changes in outer-arm galactosylation of human serum IgG N-linked oligosaccharides during aging (30) (see GLYCOSYLATION IN DISEASE section). These variations may significantly affect the bioactivity of the glycoprotein in question. For example, thyroid releasing hormone (TRH) (a tripeptide) increases the rate of secretion of TSH by hypothyroid mouse pituitaries in vitro (31). The TSH produced in response to TRH has a greater specific bioactivity than the basal TSH. Fractionation, by serial lectin affinity chromatography of the glycopeptides of TSH secreted with or without TRH-stimulation, indicated a change in the structures of TSH-associated oligosaccharides (31). It was considered unlikely that the changes in oligosaccharide structure are secondary to a change in the

polypeptide (31), raising the possibility that the increased specific bioactivity of TSH secreted after exposure of the pituitary to TRH (32) (both in vivo and in vitro) is due solely to a change in the oligosaccharides of TSH. The up (and down) regulation by glycosylation of the bioactivity of glycoproteins that interact with cell-bound receptors is becoming a recurring theme in the analysis of oligosaccharide function (49, 276a).

Glycosylation of hormone receptors may also provide a means of regulating the binding of their ligands. A powerful approach to examining the role of glycoforms in receptor function in intact responsive cells comes from the use of mutant cells with well-defined genetic defects in oligosaccharide synthesis (5). The use of wild-type and mutant cell lines (B4-2-1 and Lec 1) derived from Chinese hamster ovary (CHO) cells has allowed the effect of glycosylation of the insulin receptor to be studied (33). The mutant B4-2-1 cells transfer to proteins a "five-mannose" lipid-linked oligosaccharide ($Glc_3Man_5GlcNAc_2$-dolichol). Lec 1 cells lack the enzyme β-N-acetylglucosaminyl transferase-I (which puts on the first GlcNAcβ1→2 residue on the Manα1→3 arm of $Man_5GlcNAc_2$), an event that is necessary for subsequent normal processing to complex-type oligosaccharides. Insulin binding to B4-2-1 cells is increased, while that to Lec 1 cells decreased, compared with the wild type. The authors concluded (33) that the higher affinity in B4-2-1 cells resulted from an increased number of complex-type oligosaccharides present on the insulin receptor (i.e. increased incidence of this particular glycoform type) compared with the wild-type, and the converse for Lec 1. Interestingly, there were no differences in the binding of the insulinlike growth factor (IGF-1) to the three cell lines, raising the possibility that the binding properties of specific hormone receptors (such as for insulin), can be selectively controlled on the cell surface via the expression of distinct sets of glycoforms.

An interesting example of the functional behavior of different glycoforms comes from the studies (34) on antithrombin III (AT-III), which is a glycoprotein that inactivates thrombin and other activated coagulation proteases. The rate of protease inhibition is dramatically increased by AT-III binding to heparin. Two distinct forms of antithrombin III, designated AT-IIIα (the predominant species, 85–90%), and AT-IIIβ (10–15%), have been isolated from normal human plasma. The amino acid compositions of each form are very similar, the gross protein structures are the same, and their reactivity toward antithrombin III–specific antibodies is identical. However, the two species differ in their attached oligosaccharides and importantly, the minor form (AT-IIIβ) binds more tightly to heparin and inhibits thrombin more rapidly. Although further analysis of the oligosaccharides is still required, this result suggests that "physiological properties of plasma glycoproteins may be sensitive to subtle differences in their carbohydrate side chains" (34).

Modulation of binding properties by oligosaccharides has also been re-

ported for the binding of the glycoprotein fibronectin to gelatin (35). Collagenous proteins are involved with fibronectin in forming a fibrillar network on the surface of various cells including connective tissue cells. The presence of polylactosamine-containing oligosaccharides on the gelatin-binding domain of fibronectin results in weaker binding to the gelatin, compared with fibronectin, which carries smaller, complex-type oligosaccharides. The differential expression of these glycoforms could result in fine control of the fibrillar network as may be required, for instance at different stages of development.

A study of the rat liver asialoglycoprotein receptor demonstrates the versatility of glycosylation in diversifying a polypeptide. This receptor, which is responsible for the selective uptake of partially deglycosylated serum glycoproteins, consists of multiple polypeptide species (RHL-1, RHL-2, RHL-3) (36). The two minor forms (RHL-2 and RHL-3) are glycoforms and differ only in the carbohydrate structures that are attached to homologous polypeptide backbones. RHL-1, the predominant species, is homologous to the minor forms, but contains one major insertion of 18 amino acids near its NH_2 terminus. However, the position of one of the N-linked carbohydrate attachment sites differs from RHL-2/3. This study is important in that it suggests that the unglycosylated Asn/Xaa/Ser(Thr) polypeptide sites may be utilized to create further variants of a glycoprotein (i.e. the same oligosaccharides, but in different positions).

Control of Biological Activity through the Occupancy of Glycosylation Sequons

Support for the hypothesis that the extent of glycosylation can control the bioactivity of a polypeptide comes from the elegant studies on IgE synthesis (37, 38), which is regulated by the IgE-binding protein. Depending on the occupancy of glycosylation sequons, this regulatory protein either enhances (IgE potentiating factor), or suppresses (IgE suppressing factor) IgE synthesis.

Extrinsic tissue plasminogen activator (tPA) is a serine protease that converts plasminogen into plasmin and can thereby induce clot lysis (fibrinolysis). Its value as a thrombolytic agent (compared with plasminogen activators such as urokinase and streptokinase) lies in its clot-specific binding to fibrin. tPA is a glycoprotein of 527 amino acid residues ($M_r \sim 70{,}000$), with four potential N-glycosylation sites (39). Cells produce it, however, in two variant forms, I and II, which differ in the number of attached oligosaccharides (40). Type I tPA has three N-linked oligosaccharides while type II tPA carries only two. Both types have similar kinetic constants in amidolytic assays, using small chromogenic substrates. However, the two types do differ in the rate at which they form an active complex with fibrin, which is able to cleave

plasminogen. These kinetics are characterized by a phase transition (lag-phase), which is longer for type I tPA than for type II. When the oligosaccharides are removed, tPA cleaves plasminogen in the absence of fibrin, although the affinity for fibrin is unchanged (41). These data together imply that a physiological consequence of tPA N-glycosylation is to downregulate the tPA (i.e. to prevent tPA from converting plasminogen to plasmin in the absence of a fibrin clot). Further, the different extents of glycosylation of types I and II tPA, with the consequent difference in their relative lag-phase in the presence of fibrin, may confer a greater control and sensitivity to tPA-mediated thrombolysis (i.e. fast- and slow-acting forms). It may also be relevant that plasminogen itself also exists in two forms, 1 and 2, with either no or one N-linked oligosaccharide units attached (42). The occupancy of N-linked glycosylation sites on tPA is also associated with the efficiency of secretion (298).

Univalent monoclonal antibodies, produced from hybrid myelomas, have enhanced cytotoxic properties (43). Interestingly, a set of monovalent antibodies appears to be produced naturally via asymmetric modulation of $(Fab')_2$, as a result of glycosylation. Serum IgG from most species can be subfractionated by chromatography on a Concanavalin A agarose affinity column. The bound fraction, normally ca. 5–15% of the total IgG, contains those IgG molecules that have cytophilic potential (44) (i.e. antigen-independent activation of macrophages). Similar fractionation of the $(Fab')_2$ and Fab fragments of precipitating, and nonprecipitating antibodies (45, 46) has demonstrated that Concanavalin A retains all the $(Fab')_2$ but only 50% of the Fab from nonprecipitating antibodies. By contrast, no fragments from precipitating antibodies are retained. When the nonprecipitating antibodies are incubated with endo-β-N-acetylglucosaminidase H, they are converted into precipitating antibodies. Therefore, the asymmetry of the nonprecipitating antibody molecules is due to carbohydrate (putatively of the oligomannose or hybrid classes), which is present on only one of the Fab arms. This oligosaccharide probably affects the interaction between the Fab-combining site and its antigen and renders the molecule functionally univalent. The presence of a non–complex-type oligosaccharide on the nonbinding Fab arm of the IgG, may itself have an additional role in acting as a receptor for the serum mannan-binding protein (47), a lectin specific for mannose and N-acetylglucosamine, which activates complement through the classical pathway.

Research on the glycoprotein human granulocyte/macrophage colony stimulating factor (hGM-CSF), which regulates the growth and differentiation of certain blood cell types from progenitor cells, has demonstrated that it is crucial to measure any effects of altered glycosylation against the "native" glycoprotein in a "native" assay system. When this protein was produced from a recombinant source (in *Escherichia coli*) (48) in which no glycosyla-

tion occurs, the molecule exhibited the in vitro activities of the native form except that it failed to stimulate erythroid-burst promoting activity, suggesting that glycosylation may be essential for this activity at least, and emphasizing the range of activities of a single glycoprotein. Recombinant hGM-CSF has also been produced in yeast (49) [which essentially produces only N-linked oligomannose-type structures (51)], in Chinese hamster ovary cells (49) [which will produce hamster-specific glycosylation (2)], and in SV40-transformed COS cells (50) (see *Associations of N-Glycosylation with Tumorigenic and Metastatic Phenotypes* section for the glycosylation characteristics of transformed cells). Deglycosylation of these "native" glycoproteins in all cases resulted in a substantial increase in specific activity (approximately sixfold higher). The authors' conclusion is that "carbohydrate modification is not necessary to allow the full range of progenitor cell stimulation provided by mature hGM-CSF" (50). However, we note that this increase in specific activity is measured against an inappropriate baseline, which does in fact suggest a downregulatory role for the oligosaccharide, which may be important in a physiological context, as may be the case for tPA.

SPECIES-SPECIFIC AND TISSUE-SPECIFIC N-GLYCOSYLATION—GLYCOTYPES

Species-Specific N-Glycosylation

The species-specifity of N-glycosylation is now well-established (for a recent review, see Ref. 2), and has several important implications. In particular, since viruses utilize their host cell's glycosylation apparatus, species-specific glycosylation can influence viral tropism, as discussed in a later section HOST-CELL GLYCOSYLATION OF VIRUSES. Similarly, the expression of glycoproteins in a variety of host cells derived from different species will lead to the isolation of a polypeptide glycosylated in a manner characteristic of the species in whose cells it was expressed.

For example, a nonreducing terminal Galβ1$\rightarrow$3 residue is characteristic of serum glycoproteins of bovine and rat species [e.g. bovine prothrombin, Factor X, C1q, and fibronectin (2)]. Similarly, rat α_1-acid glycoprotein is N-glycosylated in a quite different way from human α_1-acid glycoprotein, the major difference relating to the degree of branching, fucosylation, and outer-arm decoration of the complex oligosaccharides (52). A particularly detailed study has been made of the N-glycosylation of γ-glutamyl transpeptidase (γ-GTP) expressed in liver and kidney of bovine, mouse, and rat species. Mouse kidney γ-GTP contains oligosaccharides with outer-arm fucose, a linkage that is totally absent in rat and bovine kidney γ-GTP. Rat liver γ-GTP differs from mouse and bovine liver γ-GTP in the degree of branching and

sialylation of its attached N-linked oligosaccharides (2, 53–56). The qualitative nature of these differences in glycosylation between species makes it unlikely that they principally reflect the minor difference in polypeptide structures between various species, and is taken as strong evidence for species-specific N-glycosylation.

Recently, it has been shown that an inverse relationship exists between species-specific glycosylation characteristics and the presence of naturally occurring antibodies against carbohydrate epitopes present in other species. For example, the terminal Galα1→3Gal structure is present in New World Monkeys and nonprimate mammals, but absent in humans and Old World Monkeys (57). These latter species contain high levels of natural antibody (~1% total serum IgG) against the Galα1→3Gal epitope, implying that glycoform "incompatibility" exists between the two species. The implications of such an observation for the production of glycoproteins for therapeutic use in humans are obvious (see section on *Pathological Consequences of Increased Levels of Agalactosyl IgG in Rheumatoid Arthritis*). Further, a recent structural analysis of plant glycoproteins shows that the associated oligosaccharides have the twin characteristics of a newly established family of N-linked glycans, so far only reported in plants (58). These characteristics are substitution of the pentasaccharide core [Manα3(Manα6)Manβ4GlcNAc-β4GlcNAc] by (*a*) a D-xylose residue linked β1→2 to the β-mannosyl residue, and (*b*) an L-fucose residue linked α1→3 to the reducing terminal N-acetylglucosamine residue. Since this structure contains epitopes that are highly immunogenic in mammalian species (58, 58a), the therapeutic use of mammalian glycoproteins expressed in plant cells is likely to be associated with adverse immunological reactions. Current structural analysis of the characteristics of parasite N-glycosylation suggests that similar problems ("glycoform" incompatibility and/or immunogenicity) will be encountered. For example, it has recently been shown that the parasite *Schistosoma mansoni* expresses similar antigenic determinants to its intermediate or definitive host (295). Human infection by *S. mansoni* revealed a strong immunogenicity of a surface antigen (38 kd). This protective epitope is an oligosaccharide that is expressed constitutively in its uninfected intermediate host, and is also present in many different freshwater mollusc species. The epitope was also present on the glycoprotein keyhole limpet hemocyanin, and may account for the antigenicity of this protein in many immunological studies.

There are also some examples of conserved glycosylation characteristics between species. For example, many liver glycoproteins studied from several species all appear to lack the "bisecting" GlcNAc residue in their complex-type N-linked oligosaccharides, whereas this residue is particularly prevalent in kidney-derived glycoproteins (2). Similarly, the N-glycosylation of rat and mouse thymocyte Thy-1 appears to have many features in common (10), particularly with respect to the incidence of nonreducing terminal

α-galactose residues, poly-lactosaminoglycan–type structures, and the disposition of high-mannose structures. In short, species-specific glycosylation, as well as differences in the glycosylation capacity between individuals of the same species, are now firmly established. Of greater interest may be the conservation of N-glycosylation of some peptides across species, since this could reflect important and basic conserved functions of oligosaccharides (see section on NEURAL-CELL ADHESION).

Tissue-Specific N-Glycosylation

Much structural evidence suggests that only a relatively small fraction of the oligosaccharides that an organism is capable of synthesising are associated with any particular N-glycosylation site (2, 10, 59–63). While the enzymology of oligosaccharide biosynthesis has been elucidated (1, 2a), relatively little is known about the factors that determine the set of structures associated with a particular N-glycosylation site (63). Factors that have been considered tend to fall into one of two general categories, namely, control of the glycosylation by the polypeptide backbone, and expression of a selective glycosylation capacity by the tissue synthesizing that polypeptide. It is generally considered that a polypeptide influences its own glycosylation by controlling the accessibility of the enzymes involved in glycosylation to its attached oligosaccharides. It may do this by limiting access (steric hindrance), or by restraining its oligosaccharides into particular conformations, thereby converting these into substrates for the glycosylating enzymes, or not, as the case may be (8). Extensive discussion of the control exerted by a polypeptide upon its own glycosylation can be found in Refs. 64–68.

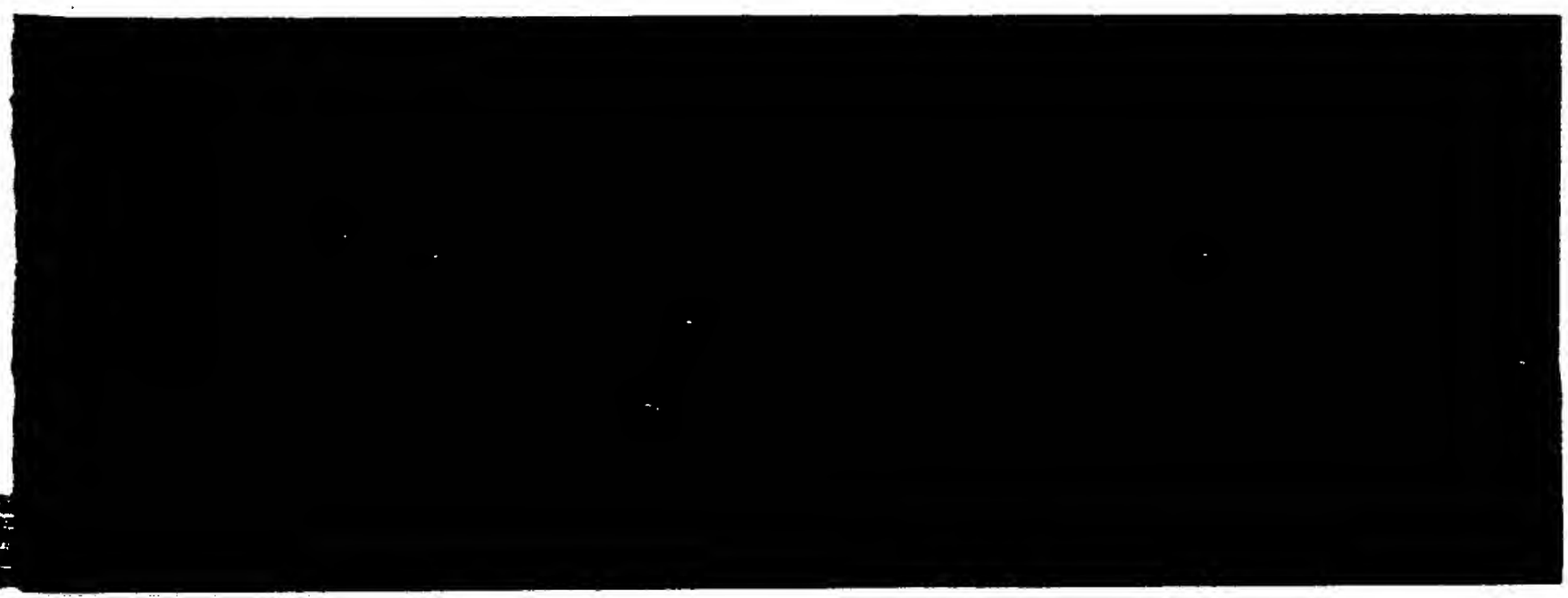
The most direct approach for determining the tissue-specifity of N-glycosylation is to compare the structure and disposition of oligosaccharides on a polypeptide when it is expressed in two or more different tissues or cell-types of the same species (2, 69–73). For example, a comparison of the glycopeptides of the HLA-DR antigen isolated from a human melanoma cell line (SK-MEL-37) and from a B-lymphoblastoid cell line originating from the same individual indicated clear differences in the N-glycosylation of the HLA-DR antigen in the two tissues, both with respect to the size of associated oligosaccharides and the extent of sialylation (69). Sialylation of murine Ia antigens has also been shown to differ between murine B-cells and murine adherent cells (70), and this difference may be important in terms of the antigen-presenting ability of the two cell-types (71).

Following an earlier report on the cell-specific glycosylation of Sindbis virus (74), it has been shown that glycosylation of one (Asn-245) of the two glycosylation sequons of the viral surface glycoprotein E1 depends on whether the virus is grown in chick embryo fibroblasts, BHK-21 cells, or Chinese hamster ovary cells (73). The glycosylation of the other site (Asn-

139) is largely unaffected by the cell-type in which the virus is grown, as is glycosylation of the second virus glycoprotein E2. In an interesting new approach, the chicken ovalbumin gene was spliced into the Herpes Simplex virus type I genome and thereby introduced into mouse fibroblast L-353 cells (75). The resulting protein was N-glycosylated at the correct sites with just oligomannose and hybrid structures in a relative ratio similar to that of native ovalbumin. However, the hybrid structures synthesized by the L-353 cells were completely sialylated and totally lacked the bisecting GlcNAc, thereby differing from those of native ovalbumin. These two examples provide strong evidence for cell-type–specific glycosylation.

Using various lectin-agarose columns (76), it has recently been demonstrated that the glycosylation of human ribonucleases from various human viscera and body fluids is organ-specific, but no structural information was obtained, nor were the ribonucleases fractionated. In an immensely detailed structural study of the N-glycosylation of γ-glutamyl transpeptidase (γ-GTP), the oligosaccharides were analyzed of γ-GTP isolated from neonatal mouse liver (53), adult mouse kidney (53), rat liver (54), a rat hepatoma cell line (AH-66) (54), bovine kidney (55), and bovine liver (2). Not only were clear-cut differences found between the same organs from different species, but also several marked differences between organs of the same species. In particular, the bisecting GlcNAc was invariably absent from the liver enzyme, but present at a high level on the kidney enzyme, irrespective of species. Further, mouse kidney γ-GTP oligosaccharides contained the Galβ1→4(Fucα1→3)GlcNAcβ1→RHdeterminant (i.e. X-antigen) (56), which is totally absent from mouse liver γ-GTP. Such studies provide strong evidence for tissue-specific N-glycosylation, but the possibility must be considered that they reflect either mutational events (77) or genetic polymorphism (78).

To avoid such difficulties, the oligosaccharide structures were determined at each of the three N-glycosylation sites of rat Thy-1 isolated from rat brain and thymus (10). There is only one structural gene for rat Thy-1, and the polypeptide of rat brain Thy-1 is known to be identical to that of rat thymocyte Thy-1 (79, 80). Moreover, previous results had shown a difference in monosaccharide composition between the two forms (81). The results of the structural analysis on the oligosaccharides therefore allowed a reconstruction of the glycoforms of Thy-1 present on rat brain and thymus, and it is clear that there is no glycoform in common between the two tissues (Figure 4). In view of the significant differences between the glycoforms, these differences probably reflect different biosynthetic events and not just the action of ecto- or exo-cellular enzymes on a common set of glycoforms. This result therefore indicates that the two tissues express quite different glycosylation capacities. However, even though Thy-1 is related to the immunoglobulin domain (82), a highly stable and conserved structure, and would therefore be expected to



have a similiar (if not identical) tertiary structure in the two tissues (Figure 4), the possibility cannot be ignored that differences in glycosylation were secondary to differences in the precise folding pathways followed by the polypeptide in the two tissues.

There have been a few reports suggesting that a polypeptide has been glycoysiated in the same way by different tissues or cell types (68, 83). None of these studies obtained sufficient structural information to confirm this suggestion (identity being defined, at best, in terms of "class" of oligosaccharide). Further, glycosylation of a polypeptide in a similar way by different tissues does not disprove a theory of tissue-specific glycosylation, since that polypeptide may have a structural requirement for a particular glycosylation pattern, irrespective of its tissue of origin.

To summarize, species-specific and tissue-specific N-glycosylation are now firmly established, and the unique glycosylation capacity of the cell has been referred to as defining its glycotype. A tissue-specific glycotype could arise in several different ways. Besides the obvious possibility of differences in the level, activity, and expression of individual intracellular glycosidases and glycosyl transferases, additional factors such as intracellular routing through the cell (84), precise topography and order of exposure to glycosidases and glycosyl transferases (85), levels and availability of necessary metabolites, and the activities of ecto- and exo-cellular enzymes, must be considered (86). An important advance in understanding the origin and control of a tissue or cellular glycotype will be knowledge of the concerted regulation of gene expression of both intra- and extra-cellular glycosidases and glycosyltransferases. Despite the use of a common biosynthetic pathway, and considerable overlap with respect to the structures they can synthesize, tissues appear to generate remarkably few glycoforms in common. The implications of this are obvious for the creation in space and time of cell-specific determinants of possible importance in intercellular interactions during development. If a glycotype serves as a statement of cellular identity, interpretation of this by other cells requires a complementary array of lectins. A better definition of the range and characteristics of mammalian lectins is needed, and would mark a major advance (87). Similarly, expression of glycoproteins of one species in another, by genetic engineering, may lead to the isolation of variants of that glycoprotein that are immunogenic in or incompatible with the original host.

HOST-CELL GLYCOSYLATION OF VIRUSES

To date no evidence has been presented that viruses encode within their genomes the enzymes required for the biosynthesis of N-linked oligosaccharides. Consequently, it is assumed that viruses carrying N-linked oligosac-

charides on their surface coat are reliant on the host cell's glycosylation apparatus. In support of this, the characteristics of viral polypeptide N-glycosylation are very similar to those of the N-glycosylation of host-cell polypeptides (see GLYCOFORMS section). For example, there is often considerable microheterogeneity at an individual site (88, 89), the structures found at a given site are influenced by both the polypeptide structure (64, 90) and the host cell (73, 74), and consequently a variety of glycoforms of each glycoprotein are produced. The structural analysis of oligosaccharides present on viral glycoproteins is complicated by the host cell dependence, and it is somewhat arbitrary to speak of specific oligosaccharide structures on a virus without considering its host.

The most detailed analysis of oligosaccharide structures present on viral glycoproteins comes from the work of Kobata and colleagues. These studies involved an analysis of the oligosaccharide moieties of glycoproteins from Sendai virus (F and HANA protein) (89) and influenza virus (BHA protein) (88), both grown in the allantoic sac of embryonated chicken eggs. Only the neutral oligosaccharides were examined since the acidic structures proved refractory to analysis (although it was established that the charge was not due to sialic acid). The neutral fraction was extremely heterogeneous, but no novel structures were reported. Other studies, although not as complete, confirm the general conclusion that the structures of viral oligosaccharides are consistent with known biosynthetic pathways of the host (91, 92).

The location and nature of oligosaccharides on viral surface glycoproteins can in some cases determine the efficiency and range of cellular infection. Since glycosylation is in part due to the properties of the host cell, the latter can therefore play an important role in defining the infective range of a virus. Sindbis virus is an alphavirus capable of infecting productively both arthropod and vertebrate cells (93). Its natural cycle of transmission involves alternate growth in mosquitoes and vertebrates, and it carries on its surface just two glycoproteins (E1 and E2), each of which have two N-linked oligosaccharides. Sindbis virus variants, with one or two additional N-glycosylation sites in E2, have been isolated (93), and the presence and location of these additional sites in each variant has been confirmed by nucleotide sequence analysis (94). The variant with one extra glycosylation site (which is invariably glycosylated), is indistinguishable from the "normal" form with respect to replication efficiency and host range. The variant with two extra glycosylation sites (both of which are glycosylated), is able to replicate only in mosquito cells and not in vertebrate cells (electron microscopic analysis revealed a failure to form the nucleocapsid). Of 15 unrestricted revertants isolated from this latter variant, all had lost the same (fourth) N-glycosylation site, as judged by nucleotide sequence analysis (93, 94). This implies that the particular oligosaccharides placed at the fourth N-glycosylation site by ver-

tebrate cells are different from the structures placed at this site by mosquito cells (and prevent nucleocapsid formation), whereas those structures added at this site by mosquito cells do not. This is consistent with structural analysis of the oligosaccharides attached to Sindbis virus glycoproteins by mosquito and vertebrate cells, which has shown that Sindbis virus grown in vertebrate cells contained complex-type oligosaccharides, whereas that grown in mosquito cells was predominantly $Man_3GlcNAc_2$ (95).

The effect of glycosylation on viral host range has also been clearly documented in the case of the influenza A virus (96, 96a). This virus binds to cell-surface receptors via hemagglutinin, a glycoprotein on its surface. In a study of the WSN strain of influenza virus, a spontaneous mutant was isolated during serial passage in Madin-Darby Bovine Kidney (MDBK) cells with a superior infective host-range. The parent and the mutant both grew equally well, and their respective hemagglutinins bound equally well to cell-surface receptors on chick embryo fibroblasts. However, during growth in MDBK cells the mutant was much more successful than the parent, due to a much greater affinity with which the mutant hemagglutinin bound to the MDBK surface. The hemagglutinin subunits (HA1 and HA2) of the two forms were compared. While the HA2 subunits appeared to be identical, the HA1 subunits were not, and the only significant detectable differences between these was the loss of an N-glycosylation site in the mutant (at residue 125) when compared to the parent, as shown by nucleotide sequence analysis. This loss of one oligosaccharide site (of the complex type, as shown by its sensitivity to Endo F but not Endo H) appears to be the major difference. Of 11 independently derived mutants, all showed this reduction in oligosaccharide content (96). It was concluded that the oligosaccharide(s) attached at Asn-125 of the parent virus hemagglutinin by MDBK cells reduced its affinity for the MDBK cell, whereas the oligosaccharide attached to this same site by chick embryo fibroblasts left the affinity of hemagglutinin for MDBK and CEF cells unaltered. These two studies show that host-cell glycosylation can clearly exert a considerable effect on the spread of viruses between different hosts.

In the case of the influenza virus, it has also been shown that the precise receptor-specificity of the viral hemagglutinin is affected by its species of origin (97). The H3 serotype, when isolated from human cells, agglutinates erythocytes carrying the N-acetylneuraminic acid $\alpha 2 \rightarrow 6$ galactose linkage but not those carrying the N-acetylneuraminic acid $\alpha 2 \rightarrow 3$ galactose linkage. The converse is true for H3 isolates from avian or equine cells. The two hemagglutinin polypeptides show 96% amino acid sequence homology, and it was considered that the differences would not necessarily affect the binding site, raising the possibility that the host-cell glycosylation may influence the hemagglutinin receptor-specifity.

Relatively subtle variations in the primary amino acid sequence of viral glycoproteins responsible for binding to target cell-surface receptors are associated with changes in the glycosylation of the polypeptides (98). The glycosylation of each hemagglutinin of 21 influenza A strains of various serotypes was compared after growth in chick embryo cells. The hemagglutinin of each strain was associated with a distinct N-glycosylation pattern (98), confirming the idea that even subtle changes in primary structure of the hemagglutinin can cause changes in the oligosaccharide chains attached to these polypeptides. Indeed, not only has serotype-specific N-glycosylation been observed, but so too has strain-specific N-glycosylation, indicating that both antigenic shift and antigenic drift are associated with alterations in viral surface oligosaccharides (99).

Further, viral surface oligosaccharides may be ligands for host cell-surface lectins, and in this way contribute to the host cell-range of that virus. For example, the paramyxovirus Sendai virus contains two surface proteins, namely the F protein and the viral attachment protein HN. The HN protein is a lectin able to recognize members of the gangliotetraose family of gangliosides (100) on its target cell-surface [it additionally possesses sialidase activity (101)]. Following binding, the viral envelope and cell-surface fuse. A temperature-sensitive mutant of Sendai virus, when grown at the restrictive temperature, carries no HN glycoprotein on its surface, and is unable to infect conventional host cells (102). However, the N-glycosylation of the F-protein makes the mutant virion a target for the asialoglycoprotein receptor of the liver, and fusion of the viral envelope with a hepatoma cell-line expressing this receptor can occur (102). In a similar fashion the binding of the HTLV-III/LAV virus to CD4+ T-lymphocytes may involve the oligosaccharides of the viral surface glycoprotein gp120 (103).

The gp120 envelope protein of the HTLV-III/LAV virus contains 24 potential N-glycosylation sites, and half its molecular weight is due to attached carbohydrate (104). It is able to bind directly to the CD4 surface-glycoprotein of T-lymphocytes (105), suggesting that it may be the viral adhesion protein. Envelope glycoprotein attachment to the CD4 molecule has been implicated in the formation of multinucleated giant cells (106). Purified gp120 is a potent inhibitor of this fusion, but recombinant gp120 fragments, immunologically indistinguishable from gp120, are not (107). These fragments are not glycosylated, suggesting that the oligosaccharides of gp120 may be important in its highly specific interaction with CD4 (107). Further evidence for this comes from the recent observation (103) that deglycosylation of purified gp120 with Endoglycosidase F causes a 50-fold reduction in its binding to CD4 and in its inhibition of cell fusion. The N-linked carbohydrates of CD4 are unlikely to be involved in binding gp120, since

soluble CD4 proteins generated using recombinant DNA techniques and lacking oligosaccharides are powerful inhibitors of HIV infection in vitro (107a).

It has been proposed that the carbohydrate side-chains on the surface of viruses may provide them with significant protection against the host's immune response during a primary infection. For example, the major immunological response in cattle to bovine leukemia virus is against carbohydrate moieties, or peptide epitopes under the influence of carbohydrate moieties (108). Further evidence for the importance of carbohydrate in directing the humoral immune response comes from a study of heteroantisera (produced in rabbits or goats) against fully glycosylated murine retroviruses or influenza virus (109, 110). These antisera were virtually unreactive against viral glycoproteins when the carbohydrate was removed from the viral surface antigens. The neutralizing antibodies, however. were a discrete subset of antibodies directed solely against polypeptide epitopes not involving, or influenced by, carbohydrates (109, 110). This is also true for the neutralizing antibodies against the HTLV III/LAV virus (107).

Carbohydrate side-chains may further assist a virus to escape from, or be resistant to, neutralizing antibodies by directly masking the polypeptide epitope for these antibodies. For example, growth of the H3 subtype of influenza virus in the presence of a neutralizing antihemagglutinin monoclonal antibody led to the selection of a variant that differed from the parent by a single amino acid substitution in its hemagglutinin (111). This substitution created an additional sequon that was N-glycosylated at the Asn, and consequently the hemagglutinin was not recognized by the monoclonal antibody. The above results raise the possibility that a general property of viral surface oligosaccharides may be to deflect the humoral immune response into the production of non-neutralizing antibodies.

In summary, the use of the host cell's glycosylation apparatus leads to the generation of virions expressing host (and therefore "self") oligosaccharides on the virus surface. One consequence of this may be to diminish an antiviral immune-response in the infected individual. Further, the carbohydrate moieties generally direct the immune response into an ineffective antibody response potentially protective to the virus (i.e. non-neutralizing antibodies). The oligosaccharides on the virus surface can influence its hostcell range. The variety of oligosaccharides attached at each site, as in the case of host glycoproteins, would create a population of viral-associated adhesion glycoproteins. Exaggerated changes in this population resulting from subtle changes in polypeptide structure would generate a range of different virions with potentially different host ranges and efficiencies of infection.

LECTIN-CARBOHYDRATE INTERACTIONS IN LYMPHOCYTE MIGRATION

The developmental history of lymphocytes involves their numerous movements around the body, each of which is necessary either for them to achieve a particular developmental stage, or to search the body for antigen. These include the movement of bone marrow–derived progenitor cells to other primary lymphoid organs, and the movement of lymphocytes through secondary lymphoid organs, together with their constant recirculation between secondary lymphoid organs and other tissues of the body (112). Each of these movements involves specific interactions between cell-surface components of the migrating cells and of the 'target' organ or tissue. There is increasing evidence that some of these components are lectins or carbohydrates (113).

Treatment of lymphocytes with exoglycosidases (114), or inhibitors of oligosaccharide-processing enzymes (115, 116) has been shown to alter their migratory properties. For example, after sialidase treatment, lymphocytes are retained in the liver and do not regain their normal migratory properties for several days (117). In a similar fashion, after a brief incubation with swainsonine, an inhibitor of oligosaccharide processing, lymphocyte migration into the spleen is reduced (116). These studies only implicate carbohydrates on the lymphocyte surface in migration, since changes in the surface carbohydrates could create new migratory properties. For instance, the neuraminidase-treated lymphocytes were probably "sequestered" initially in the liver because they are targets for the hepatic asialoglycoprotein receptor (117). More direct evidence for a positive involvement of oligosaccharides during normal migration has only recently been obtained, and this has been due in large part to the development of an in vitro system for assessing lymphocyte binding to receptors on high endothelial venules (HEV) (118).

A rat monoclonal antibody (MEL-14) has been obtained that inhibits the interaction of mouse lymphocytes with mouse peripheral lymph node HEV (119). This monoclonal recognizes a molecule on the lymphocyte cell surface that is specifically involved in adhesion to peripheral lymph node HEV. For example, pretreatment of mature lymphocytes with MEL-14 severely reduces their binding to peripheral lymph node HEV in vitro, and migration to peripheral lymph nodes in vivo, but leaves unaffected their binding to and migration into intestinal Peyer's patches. Using lymphoma cells that migrate either to peripheral nodes or to Peyer's patches, it could be shown that the MEL-14 antigen was expressed only on the former. [Normal lymphocytes migrating to Peyer's patches do express the MEL-14 antigen, but apparently do not use it for binding to the HEV of Peyer's patches (119).] Expression of the MEL-14 antigen is developmentally regulated, being absent on immature thymocytes, bone-marrow cells, and B-cell blasts from germinal centers.

None of these cell-types migrate to the secondary lymphoid organs. Further, the MEL-14 antigen is expressed at high levels on other leukocytes, in particular neutrophils, eosinophils, and monocytes (120). Attachment of neutrophils to endothelial cells can also be inhibited by MEL-14 and by the same carbohydrates that inhibit binding of lymphocytes to peripheral lymph node HEV (see later). Analysis of the MEL-14 antigen ($gp90^{MEL\ 14}$) indicated that the antigen is an 80–95-kd glycoprotein (119), whose precise molecular weight depends on the cellular source of the antigen. A hypothetical model of $gp90^{MEL\ 14}$ has been proposed, which includes ubiquitinylation and N-glycosylation of a central core polypeptide (121, 122).

While it has not yet been shown that purified $gp90^{MEL\ 14}$ is capable of binding carbohydrate, there is considerable circumstantial evidence that is has "lectin" properties. Thus preincubation of mouse or rat lymphocytes (but not frozen sections of lymphoid organs) with mannose-6-phosphate, its structural analogue fructose-1-phosphate, PPME (a phosphomonoester mannan fragment rich in mannose-6-phosphate), or fucoidin (a sulfated polysaccharide rich in L-fucose), leads to a strong inhibition of binding in vitro to peripheral lymph nodes, but not to Peyer's patches (123–126). Of various other monosaccharides, polysaccharides, or their derivatives that were tested, none showed significant inhibitory ability. Proof that the site of inhibition was at the lymphocyte cell surface came from the use of PPME-derivatized fluorescent polystyrene beads (125). Mouse lymphocytes are found to bind PPME-derivatized beads in a manner indistinguishable from their binding in vitro to peripheral lymph node HEV (127). For example, both interactions are selectively inhibited by the same set of carbohydrates, both are Ca^{2+} -dependent, and, more importantly, the ability of thymocytes, lymphoma cells, and lymphocytes to bind peripheral node HEV correlates exactly with their ability to bind PPME-derivatized beads (127). Evidence that the lymphocyte receptor for PPME-derivatized beads is related, if not identical, to $gp90^{MEL\ 14}$, includes the ability of MEL-14 to specifically inhibit PPME-bead binding, and the exact association between peripheral lymph node HEV binding, PPME-bead binding (127), and expression of the MEL-14 antigen in several lymphoma cell-lines and their variants after selection for PPME-bead binding (127). Further, MEL-14 binding to neutrophils inhibited their binding to peripheral lymph node HEV and this inhibition showed a similar sensitivity to PPME (120). However, even an excess of soluble PPME does not inhibit MEL-14 binding, implying that the "carbohydrate"-binding domain of $gp90^{MEL\ 14}$ is distinct from the MEL-14 binding site (127).

It has recently been shown that sulfated polysaccharides inhibit binding of lymphocytes to HEV of all secondary lymphoid organs by binding to receptors on the HEV (128). Different sulfated polysaccharides showed selective effects on the binding to HEV and on the entry into lymphoid organs. This

suggests that both entry and the subsequent positioning of lymphocytes within a lymphoid organ may involve a series of different sulfated molecules on the lymphocyte surface. The relationship between this system and that mediated by $gp90^{MEL\ 14}$ has not been clarified. As is discussed later, however, sulfated oligosaccharides appear to be important mediators of neural cell adhesion events, and recent data suggest that common adhesion mechanisms may be used by both the nervous and immune systems (129).

Little is known about the biochemical nature of the determinants present on peripheral lymph node HEV, which are recognized by lymphocytes. The presumption is that these HEV express a determinant containing mannose-6-phosphate, which is recognized by the $gp90^{MEL\ 14}$ on lymphocytes. Recent studies, however, suggest that sialic acid is an important component of the lymphocyte recognition determinants of both peripheral lymph node and Peyer's Patches HEV (130, 131) and particularly on the recently described endothelial cell-surface molecule ("vascular addressin"), which is suggested to be an important tissue-specific marker for lymphocyte recognition (131a). Incubation of HEV sections with the lectins *Limulus polyphemus* agglutinin, wheat-germ agglutinin, Concanavalin A, and *Dolichos biflorus* agglutinin, had no effect on lymphocyte adhesion to either HEV type, but prior incubation with a sialic acid–specific lectin, or prior treatment with mild periodate oxidation, destroyed the capacity of either type of HEV to bind lymphocytes (130). Clearly, a structural analysis of the carbohydrates present on the HEV cell surface is essential.

The mechanisms of lymphocyte migration appear to be common to several species. For example, the adhesion of lymphocytes to HEV in the rat shows strong similarities to that in the mouse. Glycoproteins have been isolated from rat thoracic duct lymph and rat lymphocytes that appear to mediate adhesion either to peripheral lymph node HEV or Peyer's patches HEV (132–134). The binding of rat lymphocytes to peripheral lymph node HEV is also selectively inhibited by mannose- or fucose-containing carbohydrates (123, 124). MEL-14 has been shown to bind to the human lymphocyte cell-surface molecule responsible for migration to human peripheral lymph nodes, further suggesting a conserved structure to this molecule (134, 135).

There is considerable evidence that individual lymphocytes can differ dramatically with respect to their migratory properties (112, 135–137). To date, no information exists concerning the mechanisms responsible for these different migratory properties. It has recently been reported, however, that intestinal intraepithelial lymphocytes express unique carbohydrates on their surface, as detected by monoclonal antibodies against CT antigens (known to be carbohydrate in nature) (138), and it was suggested that these unique structures are responsible for the specific localization of these lymphocytes.

The diversity of lymphocyte migratory patterns raises the possibility of

organ-specific immune responses to a common antigen (since organs will come to differ with respect to their lymphocyte content), and may have implications for the induction and maintenance of organ-specific autoimmune diseases. There is increasing evidence that abnormal lymphocyte migration into certain organs can lead to an autoimmune disease. For example, in acute experimental allergic encephalomyelitis (139), an animal model for multiple sclerosis, the population of lymphocytes migrating into the central nervous system does not reflect the population in the systemic side (in particular B-lymphocytes crossed the blood-brain barrier very poorly compared to T-lymphocytes) (139).

In certain chronic human inflammatory conditions, the cells of the vessels surrounding the site of inflammation are induced to differentiate so as to create venules (morphologically similar to the HEV of lymphoid organs) that are able to sustain the extravasation of lymphocytes. A particularly good example is the induction of HEV in the synovium of affected joints of patients with chronic rheumatoid arthritis (140). The synovial HEV in such patients appears to direct lymphocyte extravasation by a distinct mechanism, since the interaction between synovial HEV and exogenous human lymphocytes in vitro (140) was found not to be inhibited by MEL-14. Further, synovial HEV could not bind B-lymphoblastoid cells specific for peripheral lymph node HEV or Peyer's patches HEV. Yet the rheumatoid synovial HEV was able to direct extravasation of lymphocytes from healthy individuals as well as those from patients with rheumatoid arthritis, implying that extravasation of lymphocytes in rheumatoid arthritis is due to the induction of synovial HEV with unique lymphocyte recognition determinants, and probably not to the existence of "abnormal" lymphocytes. These two examples suggest that at least some lymphocytes in a normal immune system have special antigen-independent mechanisms for entering immunologically privileged sites (e.g. CNS and synovium). Autoimmune diseases in such sites may arise (or be perpetuated by) aberrant sequestration of these normal lymphocytes following induction of a mechanism for their egress. This may explain why many of the locally activated lymphocytes in several such diseases are not directed against any specific local antigen. The induction of HEV can be achieved experimentally using interleukin-1 (141), and interferon-γ (142), and in vivo action of tumor necrosis factor (TNF) has recently been shown to involve neovascularization accompanied by inflammation (143).

It has been convincingly shown that autoimmune disease against normal organs can be induced by modification of lymphocytes (144). Sialidase-treated lymphocytes injected intravenously into syngeneic recipients localize to the liver through a mechanism involving the asialoglycoprotein receptor. Most recover their normal migration patterns within a few days, but a subpopulation does not. This infiltrates the liver and induces an "auto-

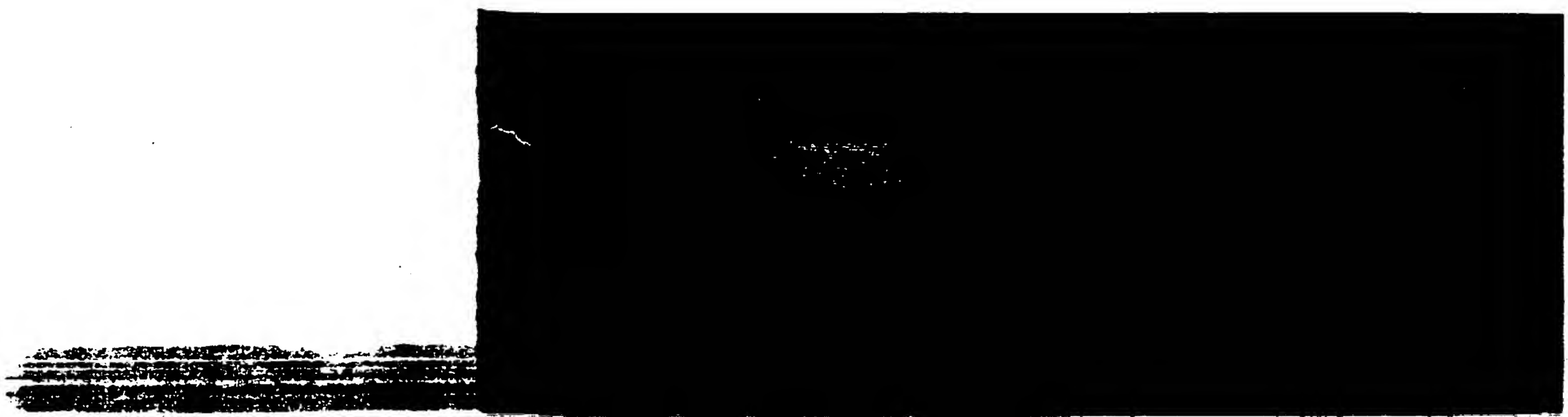
immune" disease. Similar changes in lymphocyte glycosylation may follow infection with viruses expressing a sialidase activity. By extension, any change in the glycosylation of lymphocytes that renders them ligands for any lectin of a tissue may lead to retention of those lymphocytes within that tissue, with the consequent possibility of an "autoimmune" reaction.

In summary, lymphocytes exhibit a variety of migratory patterns encompassing all or some of the organs of the body (145–149). These patterns can be altered during certain infections or disease-states, either by changes in the cell-surface properties of lymphocytes or the target tissues. The migration of a given lymphocyte depends upon the expression of certain surface receptors both by itself and by the endothelial cells of the HEV of various organs. A variety of such receptors may exist, each defining a distinct recognition system. In the case of peripheral lymph nodes, a lymphocyte cell-surface molecule with lectin-properties probably binds to complementary carbohydrates on the HEV. Lymphocytes express a variety of lectins (150), and if lectin-carbohydrate interactions are a general mechanism for lymphocyte extravasation, then tissue-specific recognition of lymphocytes by endothelial cells could arise from cell-specific glycosylation characteristics of these latter cells. Now that $gp90^{MEL\ 14}$ (possibly just one of a family of lymphocyte adhesion molecules) has been isolated, and preliminary structural studies performed on it, the way is open to isolate other lymphocyte receptors and lectins, and the targets for these on various endothelial cells. Such studies should further define the role of lectin-carbohydrate interactions, and therefore the effects of glycosylation, on organ-specific lymphocyte migration.

GLYCOSYLATION IN DISEASE

Alterations in Glycosylation—Relationships to Disease

A comparative analysis of the N-glycosylation of particular glycoproteins provides an excellent probe for acquired or inherited cell-type–specific or tissue-specific dysfunction. For example, the increased relative incidence of the serum asialo-transferrin glycoforms has been shown to accompany heavy alcohol consumption (151). Patients with nonalcoholic steatohepatitis, a liver disease that is morphologically indistinguishable from alcoholic hepatitis, did not exhibit raised levels of asialo-transferrin. Although the mechanism of production of the asialo-transferrin has not been established, it has been reported that ethanol may inhibit glycosylation of glycoproteins. Similarly, the acquired dysfibrinogenemia associated with liver disease also appears to be secondary to changes in the way fibrinogen, a liver-derived glycoprotein, is glycosylated (152). Fibrinogens from affected patients contain 1.4–3.4 more residues of sialic acid per molecule compared to normal controls, exhibit thrombin times longer than normal, and have abnormal fibrin mono-



mer aggregation. Partially desialylated fibrinogens from such patients exhibit normal thrombin times and normal fibrin monomer aggregation, indicating that the abnormality arises from increased sialylation.

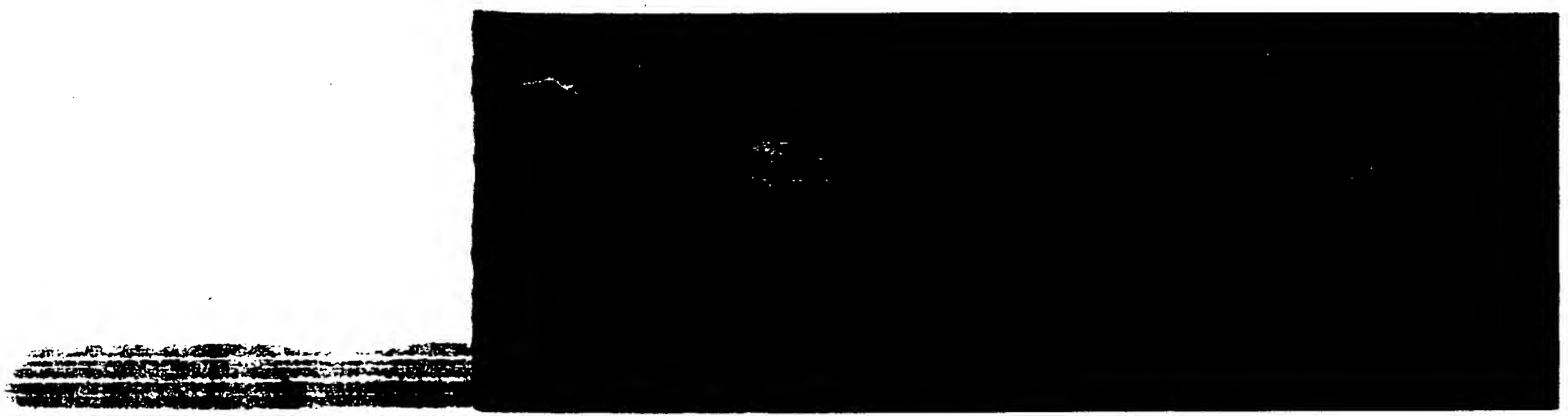
While several inherited diseases are known to be associated with abnormalities in the genes for glycosidases (6, 153, and references therein), only a limited number of genetic diseases caused by defective glycosyltransferases have so far been reported. A deficiency of GlcNAc transferase II gives rise to congenital dyserythropoietic anemia type II (153). In this disease abnormal morphologies have been detected in granulocytes, platelets, megakaryocytes, and macrophages, suggesting that the genetic lesion is not limited to erythroid cells. Since the primary pathology of this disease is related to erythroid cells, it is a good example of the difficulty in relating a particular structural change to clinical symptoms. Other examples of genetic diseases involving defective oligosaccharide biosynthesis are I-cell disease and pseudo-Hurler polydystrophy in which a deficiency of phospho-N-acetylglucosaminyl transferase activity is the primary cause (6). Cell-fusion experiments have defined complementation groups among various fibroblast cell lines derived from patients with I-cell disease and pseudo-Hurler polydystrophy. Enzyme kinetic data are consistent with the proposal that the phosphotransferase is an oligomeric protein that contains a recognition site (subunit) and a catalytic site (or subunit) that interact to recognize specifically and then phosphorylate, lysosomal enzymes. Changes in either one or both of these sites may give rise to the many disease variants, suggesting that the genetic defect involves the structural genes for the phosphotransferase and not aberrant control of its gene expression.

It may be difficult to distinguish a selective abnormality of glycosylation that is secondary to a structural change in polypeptide from one that involves primary lesions in oligosaccharide processing. For example, some variants of sucrose-isomaltase deficiency may be due to a primary lesion in oligosaccharide processing (154). Sucrose-isomaltase is an integral protein of the small intestine brush-border membrane, and can in some cases be detected immunologically in patients with a congenital deficiency of the enzyme activity. The size of the protein detected is consistent with incomplete glycosylation. Studies of a patient with primary sucrose-isomaltase deficiency demonstrated normal translation and glycosylation of the precursor with high-mannose structures, but a failure to further process the oligosaccharides with subsequent intracellular degradation of the glycoprotein and undetectable enzymatic activity of intestinal sucrose-isomaltose. In contrast, some inherited abnormalities of von Willebrand factor (vWf) associated with abnormal glycosylation may be secondary to changes in polypeptide (155). vWf is an adhesive glycoprotein involved in the binding of platelets to subendothelium after vascular injury. Initial glycosylation of nascent vWf protein has been

shown to be crucial for the successful polymerization of human vWF and its eventual secretion via Weibel-Palade bodies. The use of tunicamycin produced changes in endothelial cells that were phenotypically similar to those found in von Willebrand disease. Further studies will be necessary to confirm the role glycosylation plays in this disease.

Thyroxine-binding globulin (TBG) is an acidic glycoprotein of hepatic origin and inherited TBG variants follow an X-linked mode of inheritance. Patients with one type of TBG defect are characterized by having only a small amount of the glycoprotein in their serum (1.2% of mean normal level) (156). It does not bind thyroxine and is labile at 37°C. Consequently, these patients have high levels of circulating denatured TBG (10 × above normal). Antibodies raised against denatured TBG react with an aglycosyl TBG produced in human hepatoma cell lines in the presence of tunicamycin. This, together with the heat denaturation curve of the variant TBG, suggests the presence of glycoform subpopulations each with different thermal stabilities in patients with this TBG variant. A systemic abnormality in protein glycosylation was unlikely since the serum concentrations of six other glycoproteins were normal in affected individuals (156).

A comparative analysis of glycoenzyme thermal stability curves may provide important clues to other diseases in which abnormal glycosylation is a component. In addition, since altered activity of an enzyme involved in oligosaccharide biosynthesis could affect many different glycoproteins, the presence of several glycoenzymes showing changes in thermal stability is highly suggestive of a general glycoysIation change within their organ (or tissue) of origin, particularly in genetic diseases that are associated with just one (or a very few) loci. An analysis of the carbohydrate content of IgG from patients with cystic fibrosis has confirmed an altered monosaccharide composition as compared to total IgG from normal individuals (157). Structural analysis showed that both fucose and galactose were absent in a large proportion of the structures (unpublished data). A selective absence of galactose has already been shown to characterize the serum and synovial IgG isolated from patients with rheumatoid arthritis (RA) (158). This similar chemical difference in patients with cystic fibrosis and rheumatoid arthritis is interesting, and it is worth noting that patients with cystic fibrosis (CF) have elevated levels of circulating immune-complexes (159) as do patients with RA. Moreover, while many individuals affected with CF do not survive to the third decade, early joint complications are often reported (160). Conversely, certain patients with rheumatoid arthritis often manifest elements of a general exocrine dysfunction and have iontophoretic sweat test results at levels considered to be within the pathological range established for adults with cystic fibrosis (161).



N-Glycosylation of IgG in Rheumatoid Arthritis

Changes in the glycosylation of the serum antibody IgG in patients with rheumatoid arthritis have been well documented (17, 18, 162–164). The primary monosaccharide sequences of the oligosaccharides on IgG, and their location on the polypeptide, have been described elsewhere (17, 18). Human serum IgG carries, on average, 2.8 N-linked oligosaccharides, of which 2.0 are invariably located in the Fc (at the conserved N-glycosylation site of Asn-297). The additional oligosaccharides are located in the variable region of the light and heavy chains, with a frequency and position dependent on the occurrence of an N-glycosylation site [Asn/Xaa/Ser/Thr)] (Figure 6). Approximately 30 different biantennary oligosaccharides are found associated with total human serum IgG. These are distributed nonrandomly between the Fab and Fc. Characteristics of Fc N-glycosylation include the absence of disialylated structures, a low incidence of monosialylated ones (~10%), a low incidence of cores carrying a "bisecting" GlcNAc, and the absence of galactose on the $\alpha 1 \rightarrow 3$ arm of at least one oligosaccharide chain in each Fc. Fab

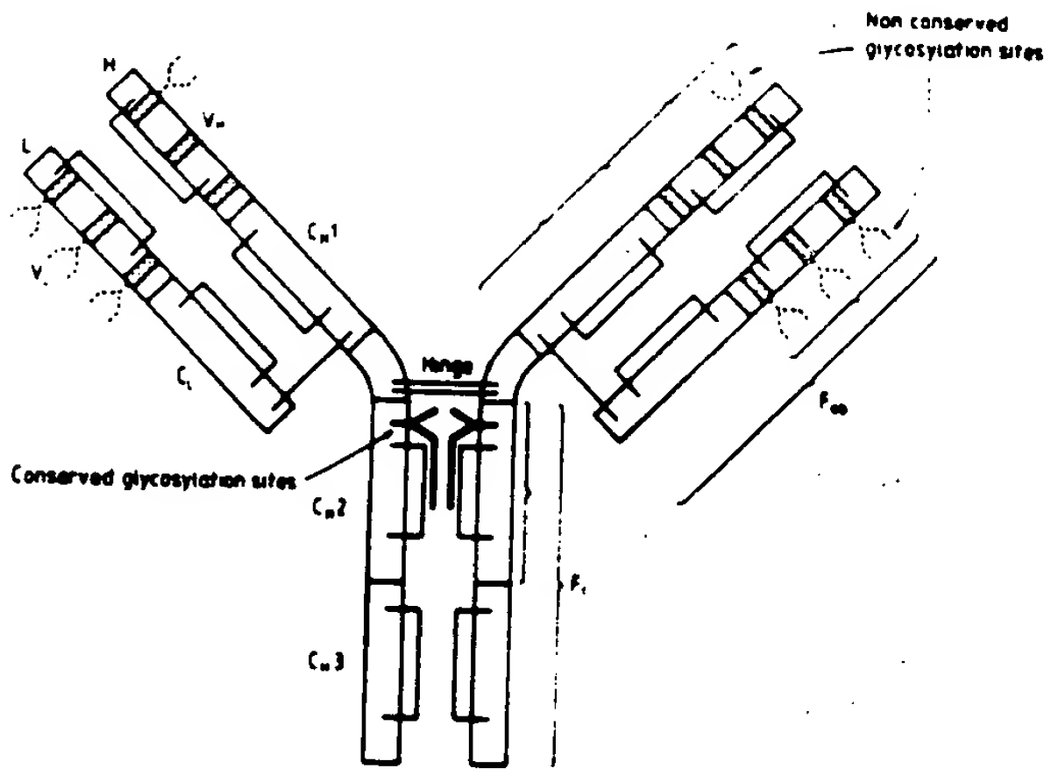


Figure 6. Schematic representation of the antibody molecule. This consists of two heavy (H) and two light (L) chains, linked by disulfide bridges (solid lines), and is divided into homologous regions of sequence (V_H, C_H1, C_H2, C_H3), each of which has an intrachain disulfide bridge. The pattern of intrachain disulfide bridging shown here is characteristic of human subclass IgG1. In V_H and V_L, the dotted segments represent the hypervariable regions of the sequence which, in the three-dimensional structure, together form the antigen-binding site. The conserved asparagine-linked biantennary complex oligosaccharide chains are attached to Asn-297 in the C_H2 domains. The number and location of Fab-associated N-linked oligosaccharides is variable and depends on the frequency with which glycosylation sequons occur in the variable regions (291).

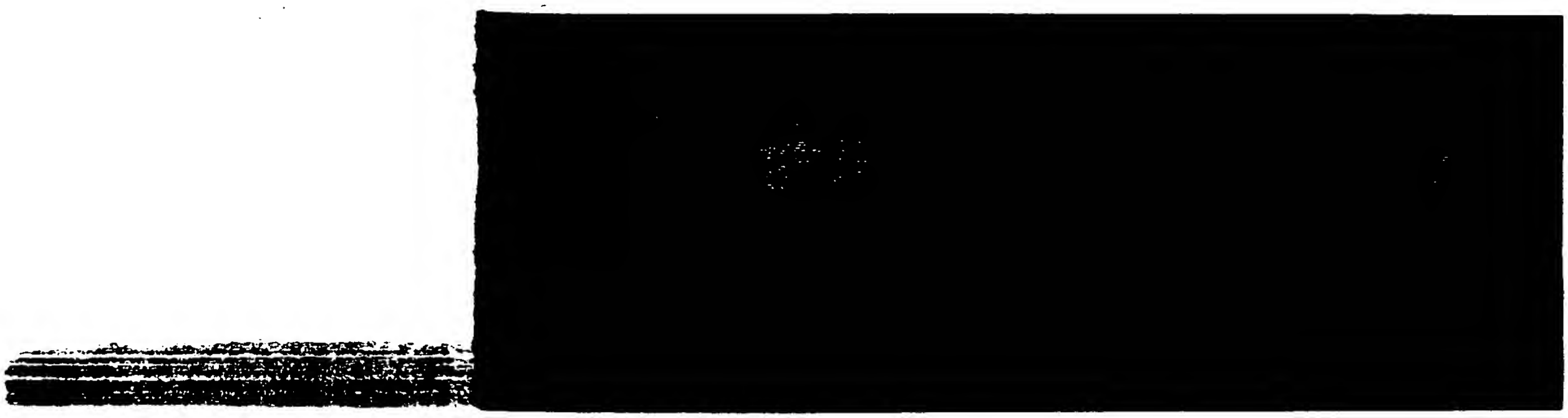
N-glycosylation is characterized by a high incidence of di- and monosialylated structures, and of cores with the "bisecting" GlcNAc residue (17, 165, 166). The large number of different structures associated with IgG is not the result of studying a polyclonal population, since a similar heterogeneity is found upon analysis of myeloma and hybridoma-derived IgG (17, 167). This heterogeneity therefore creates a very large number of glycoforms of each IgG polypeptide.

Serum IgG from patients with rheumatoid arthritis contains the same set of biantennary oligosaccharides found in normal individuals, but in very different proportions. The incidence of structures with outer-arm galactose is dramatically decreased, and the incidence of those structures terminating in outer-arm N-acetylglucosamine correspondingly increased (18, 162). A comparison of the N-glycosylation of Fab and Fc fragments derived from total serum IgG of patients with rheumatoid arthritis or from a control group shows that the decreased galactosylation found upon analysis of total serum IgG is largely due to changes in the N-linked oligosaccharides of the Fc (162). There are also quantitatively minor, but potentially significant differences in Fab glycosylation, which may be restricted to heavy-chain N-glycosylation (162).

The change in galactosylation of the serum IgG of patients with rheumatoid arthritis is not common to all other autoimmune or inflammatory disorders. On the other hand, agalactosyl IgG has been consistently found in patients with juvenile rheumatoid arthritis, Crohn's disease, and tuberculosis (163, 164).

The genetic and environmental factors leading to altered galactosylation of the N-linked oligosaccharides of serum IgG in the above diseases are at present not known. A recent report suggests that a specific galactosyltransferase is present in B-cells that transfers UDP-Gal to an asialo-agalacto IgG (168). The specific activity of the galactosyltransferase from the B-cells of such patients toward asialo-agalacto IgG was found to be reduced to 50–60% of normal controls, and the affinity of this enzyme for UDP-Gal in the B-cells of patients with rheumatoid arthritis is decreased. The galactosyltransferase deficiency also appears to be restricted to B-cells, and is not found in T-cells or monocytes (169).

The presence of agalactosyl IgG in patients with tuberculosis and the suggestion that mycobacteria are etiological agents in rheumatoid arthritis (170, 213) are consistent with previous data, which suggested that patients with rheumatoid arthritis constitute a unique group with respect to the immunogenetics and nature of their antibody response to mycobacterial antigens (171). Further, the serum of tuberculosis patients has been shown to contain self-associated IgG, and the molecular structure of IgG from tuberculosis patients (173) is similar to the "aged" IgG present in rheumatoid arthritis serum (see later). This and other findings support the suggestion that



mycobacteria, or auto-antigens that cross-react with them, may be involved in the etiology of rheumatoid arthritis (172).

The presence of the agalactosyl IgG in Crohn's disease may also be a clue to its etiology. For many years the inflammatory disorder of the bowel known as Crohn's disease was not considered to be distinct from intestinal tuberculosis. Evidence for a transmissible agent that could be an unusual type of mycobacterium continues to accumulate (174), and at present attention is focused on the cell-wall-defective forms. The finding of agalactosyl IgG in both Crohn's disease and tuberculosis may strengthen this proposed association (163).

Changes in IgG galactosylation has also been shown to be present almost uniformly in cases of juvenile onset rheumatoid arthritis, and is detectable irrespective of the mode of onset of arthritis in this younger age group (164). Serial studies among juvenile onset patients, and among adult onset patients whose clinical activity was independently assessed, have shown that the degree of galactosylation is directly related to disease activity (164). The finding that childhood and adult onset rheumatoid arthritis share the same defect in respect of the glycosylation of IgG suggests that there is a greater similarity between these two varieties of rheumatoid arthritis than has been hitherto considered. The observation that the agalactosyl IgG levels fluctuate with disease activity provides indirect evidence for a seminal role for this change in glycosylation in the inflammatory process which, in rheumatoid arthritis, is focused on the synovial tissues and results in bone erosions and joint destruction.

Pathological Consequences of Increased Levels of Agalactosyl IgG in Rheumatoid Arthritis

INFLUENCE OF N-GLYCOSYLATION ON IgG AGGREGATION Aggregated IgG is medically of importance for two main reasons. First, the immunopathology of several diseases has been attributed to the production of aggregated IgG molecules. For example, in rheumatoid arthritis, the chronic inflammation of the synovial membrane of affected joints, as well as many of the extra-articular manifestations of this disease, have been ascribed to immune complexes (either localized in the joint, or circulating) (175). Second, many IgG preparations used in immunoglobulin replacement therapy of patients with primary immunodeficiencies, and particularly agammaglobulinemias, lead to intolerance, probably due in part to the presence of aggregated IgG (176). Aggregated IgG may occur due to apolar contacts or electrostatic interactions between molecules, or may involve cross-reactivity with idiotype/anti-idiotype specificity. Interestingly, it has been found that aggregated IgG may be "dissolved" by treatment of purified IgG with mild pepsin at pH 4.0 (177).

Several reports have indicated that aggregation of human IgG into complexes of various sizes is critically influenced by the N-glycosylation of that IgG. For example, the IgG present in the intermediate complexes in the serum of patients with rheumatoid arthritis has been shown to have an unusually high sialic acid content (178–180). These immune complexes are also unusual in that they consist predominantly of IgG, implying that this molecule is both antibody and immunogen in this disease (181). Serum complexes in rheumatoid arthritis therefore probably consist of self-associated IgG molecules. In one case this sialic acid was shown to occur on light chains, and was crucial for IgG self-association, since its removal by neuraminidase treatment abolished complex formation (178). The immunogenic site on IgG has been localized to the Fc moiety, but there is no evidence for amino acid changes in the Fc of this IgG. The dramatic changes in glycosylation of the Fc associated with rheumatoid arthritis suggest that it is these changes that render the Fc immunogenic in this disease. The cryoglobulin and cold agglutinin properties of certain monoclonal IgM and IgG molecules have also been found to correlate with their sialic acid content (182–184). The IgG monoclonal cryoglobulin Ger has been particularly well studied (184). Its properties as a cryoglobulin have been convincingly shown to arise from sialylated N-linked oligosaccharides located on the Fab in the first hypervariable region of the heavy chain (at Asn 33). Taken together, these studies imply that N-glycosylation of the Fab of human IgG can exert an important influence on IgG aggregation/solubility.

There are now data that suggest that the aggregation/self-association of IgG involves a unique subpopulation of molecules (166). The N-glycosylation of this subpopulation is essentially normal in so far as it carries the same set of oligosaccharide structures as monomeric IgG, but it is characterized by a high level of Fab N-glycosylation and the particular prevalence of NeuNAcα6Galβ4GlcNAcβ2Manα6 (NeuNAcα6Galβ4GlcNAcβ2Manα3) Manβ4GlcNAcβ4GlcNAc on its Fab. Human plasma IgG, which contains on average 2.8 N-linked oligosaccharide chains per mole of IgG, can be fractionated into monomeric, dimeric, and aggregated forms. A quantitative analysis revealed that aggregated IgG carries on average 3.5 N-linked oligosaccharides per molecule, while monomeric IgG carries 2.2. Human serum IgG has 2.0 oligosaccharide chains on the Fc and any others are on the Fab. Hence, the aggregated form carried an average of 0.8 oligosaccharide chains on each Fab, while the monomeric form carried 0.1. This conclusion was supported by the increased incidence of disialylated oligosaccharides in the aggregated IgG, since these structures occur only on the Fab of human IgG (17, 162, 165). The ability to dissolve aggregated IgG by mild pepsin treatment indicates that molecules when aggregated must be uniquely sensitive to pepsin. There is a decrease in disialylated structures in the total IgG after pH 4.0/pepsin treatment, suggesting that the IgG aggregates were not "mono-

merized" but proteolytically degraded (166). Since dimers appear to be resistant to proteolysis, the sensitivity of the aggregates either results from the difference in the sequences of the Fab sugars or from conformational changes induced in the IgG molecules during aggregation.

Further evidence for the role of glycosylation in the aggregation of IgG comes from a study of the monoclonal antibody MRC Ox45 in which it has been shown that a subpopulation of the molecules can be induced to self-associate (166). This monoclonal antibody consists of a mixture of some molecules that carry an N-linked oligosaccharide in the Fd fragment, and some that do not (185). Since there are no amino acid differences between those IgG molecules carrying a Fab oligosaccharide and those lacking one, either the individual cells within this clonal population differ with respect to their N-glycosylation capacity, or the cells are capable of partial (i.e. "facultative") N-glycosylation. The self-associating Ox45 monoclonal antibody molecules were found to have a much higher content of disialylated oligosaccharides than the monomeric ones. Further, the relative incidence of neutral, monosialylated, and disialylated oligosaccharides in the self-associated IgG was even higher than in the $F(ab')_2$ of the total IgG (i.e. monomeric and self-associated forms). From these results, it was concluded that the self-associating forms are those molecules that contain Fab N-glycosylation (166).

To date, the direct evaluation of the N-glycosylation of IgG in the IgG-IgG self-associated complexes present in patients with rheumatoid arthritis without hyperviscosity syndrome has not been possible, since their free level in serum is generally too low for isolation in significant quantities (185). This low level is probably a consequence of the ability of human erythrocytes to bind and clear immune complexes and the capacity of this system is such that the steady-state level of free immune complexes in serum is never high (186). Further, autoreactive self-associated IgG produced in the synovium may not reach the peripheral blood in significant quantities, either because of in situ precipitation, or dilution-dependent dissociation in serum (187, 188). However, data on the carbohydrate composition of IgG present in the intermediate complexes isolated from the serum of patients with rheumatoid arthritis and with hyperviscosity syndrome invariably show an increased level of sialic acid (usually Fab-associated) (178, 179) as compared to normal serum IgG, and decreased levels of Fc galactose content (178). Together, these observations suggest that immune complex formation in rheumatoid arthritis involves both a particular Fab N-glycosylation and agalactosyl structure in the Fc. The molecular mechanisms whereby these two factors combine to cause IgG self-association are currently being explored.

In conclusion we again emphasize that a single amino acid sequence can be diversified, through N-glycosylation, into a set of "glycoforms" (i.e. glycoproteins sharing an identical polypeptide but differing with respect to the

structure, location, or incidence of individual oligosaccharides). This is particularly true in mammalian systems, and IgG is no exception. Of the 30 or so different oligosaccharide structures found on total human serum IgG, most are present on monoclonal antibodies. The number of potential glycoforms that can be generated from each monoclonal IgG polypeptide is therefore considerable, and the number of unique IgG glycoproteins in serum potentially vast, and some of these have different physical properties. In the case of both serum IgG and a mouse monoclonal antibody, those IgG molecules that aggregate carry a different relative proportion of this same set of oligosaccharides as compared to those IgG molecules that do not, and hence constitute a distinct set of glycoforms. Given the pathological consequences of IgG aggregate formation in vivo, these results support the hypothesis that alterations in the relative incidence of individual IgG glycoforms may be important in certain IgG solubility-related diseases.

BIOLOGICAL ACTIVITY An understanding of the functional role of the Fc oligosaccharides comes from studies using aglycosyl monoclonal IgG's produced in the presence of tunicamycin (189–191). The aglycosyl molecules retain the properties of the normal glycosylated molecule in respect of the binding of antigen, protein A, and C1q, and also with respect to C1 activation. However, the resistance to protease digestion is reduced, there is loss of binding to monocyte and macrophage Fc receptors, the ability to induce cellular cytotoxicity is reduced, and complexes with antigen fail to be eliminated rapidly from circulation. The carbohydrate chains have also been shown to be required for another biological function of IgG antibodies, namely feedback-immunosuppression (191). It is interesting to note that the carbohydrate-dependent functions of the IgG all involve interactions with cellular bound receptors, whereas fluid-phase reactions are unaffected. Obviously the actual activity of IgG in any carbohydrate-dependent function is a "composite activity" due to the heterogeneity of the glycosylation of the Fc. This places in context the pathological consequences of relative changes in the incidence of individual IgG glycoforms in a variety of disease states.

Disease states that are characterized by IgG molecules with exposed nonreducing terminal N-acetylglucosamine residues may have an immunopathology due to the exposure of a "latent" IgG function. Diseases associated with raised agalactosyl IgG levels (rheumatoid arthritis, Crohn's disease, and tuberculosis) are characterized by inflammatory tissue damage, fever, and weight loss. On the other hand, sarcoidosis and leprosy, which exhibit normal levels of agalactosyl IgG, are characterized by granulomas and massive macrophage activation, both via gamma-interferon and via a positive autocrine feedback loop involving 1,25 dihydroxyvitamin D_3 formation by the interferon-activated macrophages (192, 193). Such macrophages are

known to be primed for release of tumor necrosis factor. Yet patients with sarcoidosis or leprosy show little or no weight loss, fever, or necrosis secondary to endothelial cell damage and microcapillary thrombi. Thus, TNF/cachectin appears not to be released in these latter diseases.

High release of TNF could indeed account for several clinical features of tuberculosis and rheumatoid arthritis that are absent in sarcoidosis (192). TNF is now known to be identical to cachectin, which causes weight loss (143 and references therein). It is also a pyrogen, and the major mediator of endotoxic shock, which is characterized by diffuse intravascular coagulation and hemorrhagic necrosis in the gut. Effects of TNF on the vascular endothelium lead to a loss of its normal anticoagulant properties, and release of TNF is often accompanied by the release of interleukin-1. Much of the necrosis in TB can therefore be attributed to microvascular thrombi, such as TNF will evoke. It has been demonstrated that live virulent *Mycobacterium tuberculosis* can substitute for endotoxin in triggering TNF release (192). Consequently, the ability of the *M. tuberculosis* organisms to trigger the activated macrophages to produce TNF in tuberculosis (but not in sarcoidosis) may give rise to the individual disease characteristics.

In vitro, TNF has been shown to inhibit endothelial cell proliferation in cultures and consequently it has been suggested that in vivo it may inhibit, for example, tumor neovascularization. However, recent data have demonstrated that in vivo TNF stimulates vascularization (143). This neovascularization is accompanied by a leukocyte infiltration (i.e. inflammation). Other well-established angiogenesis factors induce capillary vessel formation in the absence of an inflammatory response. TNF is readily detected in the synovial fluid of patients with rheumatoid arthritis (194). Further, established synovitis in patients with rheumatoid arthritis is associated with neovascularization. It has recently been proposed that agalactosyl IgG itself is a potent stimulator of TNF release from activated macrophages (163). This may be a consequence of IgG bound to the macrophage Fc-receptor also binding via its exposed nonreducing terminal GlcNAc residues either to some macrophage GlcNAc-binding receptor (195), or to the GlcNAc-binding CR3 receptor (196). These GlcNAc-receptors appear also to bind lipopolysaccharide or lipid X with subsequent TNF release.

"AGED" IgG The galactosylation of the N-linked oligosaccharides of human serum IgG is an age-related molecular parameter (197). The extent of galactosylation was found to vary parabolically with age. Immunological competence is known to vary with age, and interestingly, these age-dependent variations are also often parabolic in nature. For example, both antibody response and avidity increase during postnatal development, reach a maximum in adulthood, and then decline during senescence (198). Cellular

changes (both helper and suppressor T-cell levels) appear to account for most of these variations in immunological competence (198, 199). Surviving older humans have an increased frequency of auto-antibodies and rheumatoid factor, as well as an increased frequency of idiopathic paraproteinemias (200). The heterogeneity within age groups may be due to intrinsic differences in genetic endowments, or may reflect the impact of extrinsic factors. The natural variation in galactosylation of IgG with age, together with the observation that serum IgG from patients with rheumatoid arthritis has a similar clearance rate to that from much older normal individuals (173), raises the possibility that one of the lesions in rheumatoid arthritis may be an accelerated "aging" of the immune system. Indeed, age-related variation in oligosaccharide-mediated functions may, in some cases, be the basis for the age-association of certain diseases. Certainly more studies addressing this interesting topic are needed.

Molecular-Mimicry by Environmental Pathogens of Endogenous Carbohydrate Epitopes

Biological mimicry of endogenous products is well known (e.g. morphine mimics endorphin). A similar situation may account for the immunological effects of various peptidoglycans. For example, there are structural similarities between sleep factor (GlcNAC.1.6 anhydro-MurNAc-Ala-γ-Glu-dap-Ala), which regulates the balance of rapid eye movement (REM) and slow wave (SW) sleep, and peptidoglycan fragments from cell walls of gram-negative bacteria, which are immunoadjuvants and also somnogenic [e.g. GlcNAc muramyl dipeptide (GMDP), and muramyl dipeptide (MDP)] (201–203). Interestingly, muramyl peptides (MP) can induce the synthesis and release of interleukin-1 from leukocytes as well as from brain astrocytes (204). It has been proposed that interleukin-1 contains an MP-like structure since it cross-reacts with an antibody to MP (i.e. the antibody against MP also blocks the activity of interleukin-1). Interleukin-1 has somnogenic and pyrogenic activity, and probably contributes to the subjective feeling of sleepiness that often accompanies infectious disease (201). Obviously then, antibodies to GlcNAc-containing epitopes present in the environment, which cross-react with endogenous epitopes, may have both immunological and neurological effects.

The immune response to terminal GlcNAc may also be the basis for bee venom allergies. Recent sequence analysis of the N-linked oligosaccharide moieties of allergenic glycoproteins present in bee venom has demonstrated the presence of nonreducing terminal N-acetylglucosamine and fucose residues (205, 206, 206a). These N-glycans have been shown to be specifically recognized as epitopes by IgE antibodies from allergy sera (206). Interestingly, administration of bee venom prevents the arthritic syndromes in

adjuvant-induced experimental arthritis (using mycobacteria), and is effective in the treatment of rheumatoid arthritis in humans (207–211).

Terminal GlcNAc-residues are relatively rare in mammalian tissues. If when present they have a regulatory function, then antibodies to cross-reactive GlcNAc-containing epitopes present in the environment, particularly the adjuvant bacterial components (GlcNAc contain peptidoglycans), would have interesting autoimmune effects. IgG is unique among circulating glycoproteins in having a naturally occurring low, but still significant, basal level of glycoforms expressing oligosaccharide chains terminating in GlcNAc (unpublished results). A number of cellular receptors for GlcNAc-terminating sugar chains have been reported (212). These may be functionally important in the recognition of peptidoglycan structures of bacterial cell walls, in tumor surveillance (see later), and in macrophage recognition of cells undergoing programmed cell death (apoptosis). Immunopathological consequences of IgG molecules presenting an abnormally high concentration of peripheral GlcNAc residues can be understood in terms of a disruption of a stable network of interacting cells and receptors for the GlcNAc epitope. For example, antibody to terminal GlcNAc might have some of the properties of rheumatoid factor. Indeed, it has recently been reported that mice immunized with the peptidoglycan/polysaccharide complex of Group A streptococci can be used as a source of monoclonals binding to the terminal GlcNAc residues situated in the C_H2 domain of serum IgG isolated from rheumatoid arthritis patients (214). This study is important since Group A Streptococci, which are associated with rheumatic fever, appear to be able to evoke formation of antibodies that will bind to an epitope on the agalactosyl IgG present in rheumatoid arthritis. The Group A Streptococci peptidoglycan is rich in GlcNAc and patients with rheumatic fever are known to have raised levels of antibody to GlcNAc (292). Correspondingly, patients with rheumatoid arthritis have recently been shown to have raised levels of antibodies that bind to the Group A Streptococci (293, 294).

In summary, autoimmunity against endogenous carbohydrate epitopes may have profound effects on a variety of physiological processes. (Individual genetic endowments obviously determine how we "respond" to environmental carbohydrate epitopes.) It will be interesting to see if future research supports the idea that groups of diseases (e.g. rheumatoid arthritis, tuberculosis, Crohn's) are indeed related by a common etiology, although clinically perceived as different diseases. In addition, much may be gained by asking the question "why do certain people not develop a particular disease?", rather than from the opposite and conventional approach to etiology (215). For example a striking negative association exists between rheumatoid arthritis and schizophrenia (215). Indeed at one time it was believed that the two diseases were mutually exclusive. It has recently been proposed that the

balance of REM/SW sleep affects the tendency to hallucinate (216), and that this balance is regulated by the sleep factor containing terminal GlcNAc (201–203).

Pathological Consequences of Naturally Occurring Anti-Carbohydrate Antibodies

It has been reported (57, 217, 218) that all human sera contain large quantities (up to 1% of all circulating IgG) of a naturally occurring antibody that binds to the antigenic epitope Galα1→3Gal. Low levels of Galα1→3Gal containing glycosphingolipids have been found on human erythrocytes and may function as senescence antigens. These epitopes are exposed upon aging of the erythrocyte and serve as binding sites for the naturally occurring anti-Galα1→3Gal antibody. Inappropriate exposure of these cryptic antigens in various hematological disorders leads to premature destruction of erythrocytes and has been directly implicated in the premature destruction of erythrocytes in sickle cell anemia (217, 218). An interesting inverse species-evolutionary relationship exists between the expression of glycoconjugates containing Galα1→3Gal epitopes and the natural occurrence of this anti-Gal antibody (257). In view of the species specifity of N-glycosylation, the presence of naturally occurring antibody in human serum against carbohydrate epitopes on circulating glycoproteins in New World monkeys and nonprimate mammals (e.g. rat, rabbit, cow, etc) obviously may limit the use of human glycoproteins expressed in cell-lines derived from these species.

Associations of N-Glycosylation with Tumorigenic and Metastatic Phenotypes

In many instances the pattern of tumor spread cannot easily be explained by direct extension followed by diffuse or hematogenous spread. The rules governing the peculiar tropic behavior of many tumors are not yet fully understood. However, the expression of specific oligosaccharide structures on cells may contribute to their tumorigenic or metastatic behavior. For example, a comparative analysis of the cell-surface oligosaccharide structures present on baby hamster kidney cells (BHK) following viral transformation by either the Rous sarcoma virus or the polyoma virus has demonstrated an increase in Asn-linked complex-type oligosaccharides that contain an N-acetylglucosamine residue linked β1→6 to mannose (92, 219, 220). Interestingly, the changes were relative (i.e. not qualitative) and found to be secondary to an increased relative activity of GlcNAc transferase V (219). More recently it has been demonstrated that the presence of leukocyte agglutinin (L-PHA) binding β1→6 branched N-linked oligosaccharides on an individual cell-surface glycoprotein (gp130) correlates with intracellular levels of GlcNAc transferase V, and in a linear way with the metastatic potential of

tumor cell lines, but not tumor formation per se (221). Both selection for and against metastatic potential was associated with an increase and decrease, respectively, in the expression of such N-linked oligosaccharides on gp130. These results imply that a shift in the glycoform population of gp130 (or perhaps all glycoproteins) on the tumor cell surface toward those with $\beta 1 \rightarrow 6$ linked branching of the N-linked oligosaccharides is associated with a corresponding increase in the metastatic potential of that cell. Presumably the actual metastasis of a particular tumor cell would occur once a critical threshold of the appropriate glycoform population(s) had been achieved. That is, qualitative changes in a biological property (metastatic versus nonmetastatic) could be achieved by a quantitative change in the activity of GlcNAc transferase V. The analysis of glycosylation mutants of highly metastatic tumor cell lines has shown coordinate changes in the activity of a number of the GlcNAc transferases, suggesting a regulatory mechanism that controls sets of transferases (221). These same authors have also demonstrated that tumors are heterogeneous for the metastatic phenotype, and that a subpopulation of cells expressing a high density of the "metastatic" glycoforms has a selective metastatic advantage over the cells expressing a lower density of "metastatic" glycoforms on their cell surface (221).

The predictive value of lectin binding in disease diagnosis and prognosis can be extended to the detection of carbohydrate expression in paraffin-embedded sections of primary tumors. Recently a lectin from *Helix pomatia* has been found to bind to a subpopulation of breast cancer cells associated with metastasis to local lymph nodes (222). Paraffin sections up to 20 years old were analyzed by lectin binding, and statistical associations with lymph-nodes stage, locoregional recurrence, and survival were shown.

The ability of host natural killer (NK) cells to limit metastatic spread of tumor cells may also be dependent, in part, on the density of specific oligosaccharide structures on the tumor cell surface. The use of glycosylation mutants and specific inhibitors of N-linked oligosaccharide processing have demonstrated that natural killer cells have the ability to recognize and lyse targets depending upon the type of N-linked oligosaccharides present on the target cells (221, 223–227). In particular, cells expressing high densities of high-mannose-type, hybrid-type, and incomplete complex-type (i.e. exposed GlcNAc) N-linked oligosaccharides are more sensitive to natural killer cell lysis than cells expressing completely processed sialylated complex-type oligosaccharides that appear to escape natural killer surveillance.

Glycoproteins secreted eutopically and ectopically by tumor cells in vivo generally reflect the tumorigenic process per se. The carbohydrate moieties of these glycoproteins can be useful epitopes for tumor diagnosis. Fucosylated variants of α-fetoprotein (AFP) have recently been described that appear to be good markers for hepatocellular carcinoma, and helpful in the follow-up of

patients with benign liver diseases (228–231). These variants can be detected by their reactivity with *Lens culinaris* agglutinin using lectin-agarose affinity chromatography. In the rat, ectopic expression of GlcNAc transferase III in hepatoma cells leads to the biosynthesis of γ-glutamyltranspeptidase (γ-GTP) containing "bisecting" complex-type N-linked oligosaccharides (54). The enzyme produced by normal rat liver as well as other nonmalignant diseased livers does not contain this tumor epitope. Interestingly, this change is not tumor-specific when considered in the context of organ-specificity of N-glycosylation as γ-GTP produced normally by rat kidney contains "bisecting" complex-type structures (232). Affinity chromatography of the desialylated human serum γ-GTP on a *Phaseolus vulgaris* erythroagglutinin lectin-agarose column has recently been used to confirm this structural change in patients with primary hepatoma (233). Structural characteristics of the sugar moieties of the glycohormone hCG derived from placenta, hydatiform mole, invasive mole, and choriocarcinoma further demonstrate the value of analyzing eutopically secreted glycoprotein from tumors. Both choriocarcinoma and invasive mole ectopically express GlcNAc transferase IV, and consequently produce tumor-specific glycosylation variants of hCG (234–237). The hCG molecules that contain this epitope can be detected by their ability to bind to a *Datura stramonium* agglutinin-agarose column (237). Interestingly, there is no overlap of oligosaccharide structures between the tumor-produced and normal tissue-produced hCG.

The carbohydrate analysis of ectopically secreted glycoproteins has also demonstrated the uniqueness of oligosaccharide structures produced in tumors when compared to the native forms (238, 239). A comparative analysis of human parotid α-amylase from a lung bronchioloalveolar adenocarcinoma and an ovary papillary cystadenocarcinoma showed no oligosaccharides in common between the native and cancer-derived forms. The structures of asparagine-linked sugar chains were the same in both the tumors, and were incomplete in comparison with those of the parotid-derived amylase (239). Whereas a number of the structures found on hCG derived from choriocarcinoma have not been found elsewhere, the structures present on the tumor-secreted α-amylases have been found on native glycoproteins (18) (e.g. human IgG).

NEURAL CELL-ADHESION—REGULATION OF CARBOHYDRATE STRUCTURES INDEPENDENT OF POLYPEPTIDE BACKBONE

The discovery of cell-surface glycoproteins involved in cellular adhesion has opened the way for the analysis of the molecular events involved in this important developmental process (240). These molecules have been func-

tionally defined as cell adhesion molecules (CAMs) since they influence the specific adhesion of cells as judged by in vitro assays. Two primary CAMs, which are expressed in early embryonic cells and also in the adult central nervous system (CNS), are the neural cell adhesion molecule (N-CAM) and the liver cell adhesion molecule (L-CAM). These are differentially expressed at known sites of embryonic induction (241).

The best-characterized CAMs are those involved in the embryonic development of the mouse CNS. Four such glycoproteins are now known to mediate (Ca^{2+}-independent) adhesion among different neural cell-types at different developmental stages. These are the N-CAM (a glycoprotein that occurs in the adult CNS in three forms [of 180, 140 and 120 kd, which all derive by alternative RNA splicing from a single structural gene (242)], the neuron-glial adhesion molecule(s) J1 (243), the myelin-associated glycoprotein [MAG, occurring in two forms of 67 and 72 kd (244)], which mediates neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte adhesion (245), and the L1 glycoprotein(s) implicated in neuron-neuron adhesion (246).

The above four cell adhesion molecules are all N-glycosylated, and their N-linked oligosaccharides have been implicated in the adhesion events that they mediate (247, 248). For example, the sialic acid residues have been shown to be important for modulation of the homophilic binding of N-CAM molecules (249), and differences occur in glycosylation between the embryonic and adult forms, particularly with respect to the extent and nature of sialylation of the N-linked oligosaccharides (250). Further, the four glycoproteins (i.e. N-CAM, J1, MAG, L1) appear to share a common carbohydrate epitope, as defined by the anti-carbohydrate monoclonal antibodies L2 and HNK-1 (246, 251) (the latter was originally raised against a human natural killer cell line).

Molecules that contain the L2/HNK-1 carbohydrate epitope make up the L2/HNK-1 family of cell-adhesion molecules. Further, since only some of the polypeptides of a particular adhesion molecule express the epitope, the expression of the carbohydrate structure seems to occur independently of the protein backbone (252). The importance of a "cell-adhesion family" lies in the finding that molecules expressing the epitope appear to have similar physiological roles and there are hints that the epitope itself may be involved in cell adhesion. A second cell-adhesion family is characterized by the L3 carbohydrate epitope (252, 253). Members of this family include some members of the L2/HNK-1 family (i.e. L1 and MAG), as well as distinct molecules such as AMOG (glial cell-adhesion molecules) (253).

Peripheral neuropathies are often associated with patients having monoclonal IgM due to plasma cell abnormalities (254, 255). The IgM from some of these patients has been shown to bind to the glycoprotein MAG of the L2/HNK-1 cell-adhesion family, and also to an acidic glycolipid present in

the ganglioside fraction of the human peripheral nervous system (256). HNK-1 reacts with the same acidic glycolipid antigen isolated from the human peripheral nerve, which has recently been characterized as sulfate-3-GlcAβ(1→3)Galβ(1→4)GlcNAcβ(1→3)Galβ(1→4)Glc(1→1)-ceramide (255). The presence of a 3-sulfoglucuronosyl residue in the lipid was found to be essential for HNK-1 reactivity. The HNK-1 reactive carbohydrate epitope on the cell-adhesion glycoproteins has not been characterized, but glucuronic acid has been reported to be present on the N-linked glycans of the HNK-1 reactive ependymin glycoproteins (257), and most adhesion molecules are thought to contain sulfated oligosaccharides. Ependymins are a family of extracellular glycoproteins present in goldfish and mammalian brain that have been shown to form an extracellular matrix, and have been implicated in events that lead to synaptic changes associated with learning and the processes of neural regeneration (257).

Cell-adhesion molecules may play important roles in the specification of cell contacts not only between neighboring cells, but also between the cell surface and the extracellular matrix, the components of which may also be modulated during neural (and non-neural) development. Thus, a neural proteoglycan containing an active chondroitin sulfate species and the HNK-1 epitope has been found to interact with a neuron-glial adhesion molecule, cytotactin, found in the extracellular matrix (258). Moreover, N-CAM, also with the HNK-1 epitope, is known to bind to heparinlike molecules in the cell substratum in developing chick nervous tissue (259).

Recent studies on the glycoprotein Po suggest that it exists as a heteroglycodimer complex (i.e. a complex of two different Po glycoforms). Po is the major protein constituent of myelin in the peripheral nervous system of mammals (260, 261). The structure of the extracellular domain of Po suggests that this protein may mediate the association of extracellular membrane surfaces in myelin (260). Homophilic or "self-adhesive" properties have been proposed for this glycoprotein, and are thought to derive from hydrophobic interactions involving the peptide or interactions between facing sugar moieties (260). Po has only one glycosylation site yet expresses both the L2/HNK-1 and L3 carbohydrate epitopes (262). Carbohydrate analysis of Po shows that 50% of the oligosaccharide chains contain sulfated residues (a component of the L2/HNK-1 epitope), and 50% contain sialic acid (a component of the L3 epitope). The presence of sialic acid and sulfate on a single oligosaccharide chain appears to be mutually exclusive, since no structures containing both sialic acid and sulfate were found (unpublished data). Since each Po molecule has only one attached oligosaccharide, and since only 50% of the Po molecules isolated by using an L-2 monoclonal antibody affinity column carries oligosaccharides recognized by that monoclonal antibody, each antibody-positive Po molecule must be associated with at least one Po molecule that is nonreactive and contains a different oligosaccharide. Homo-

philic self-association of a glycoprotein may therefore be a misnomer and conceptually inaccurate unless the definition of a cell-adhesion molecule includes a set of glycoforms. Such considerations are now implicit in certain recent models of cellular adhesion (262a).

Neural adhesion molecules may play a role in governing the patterns of development and distribution of both nervous and immune systems of cells. The HNK-1 epitope has recently been reported to be present on avian lymphocytes of both B and T lineages (129). Expression of this epitope during ontogeny revealed unique developmental patterns for cells of the two lymphoid pathways. Antiserum reactive with the protein part of chicken myelin-associated glycoprotein was shown to react with material in membrane extracts of HNK-1-positive thymocytes, suggesting that this nerve cell component is expressed on cells of the immune system (129). The presence of this "neural adhesion" molecule on lymphocytes may be important in the binding of lymphocytes to the high endothelium of venules. The proposal that the lymphocyte "homing" molecules, which direct the traffic of specialized subpopulations of lymphocytes, may also be utilized as neural adhesion molecules, is exciting (129) and may be important in our understanding of the mechanism of vascular egress by immunocompetent cells, and entry into the brain during multiple sclerosis and experimental allergic encephalomyelitis (263). (The antigen-independent egress of lymphocytes into the synovium in rheumatoid arthritis (140) has been discussed in LECTIN-CARBOHYDRATE INTERACTIONS IN LYMPHOCYTE MIGRATION).

Endothelial cells are induced by astrocytes (264) to form highly impermeable tight junctions, an important purpose of which is to allow a discrete internal environment to be maintained within the CNS (blood-brain barrier). Little is known about the glycosylation pattern of proteins in the cerebrospinal fluid (CSF) compared with those in serum. This is largely because the protein concentration in CSF is very much lower than that in serum, and the volume available for analysis is generally small. However, a study using serial lectin-agarose chromatography of the ribonucleases from human CSF has shown that their glycosylation is different from that of ribonucleases in human serum (76). This raises the possibility that transport across the blood-brain barrier may involve only certain glycoforms of ribonucleases or that there is modification of the oligosaccharide portion of some ribonucleases associated with this transport (265). Alternatively, if the ribonucleases are synthesized within the central nervous system (CNS), then in view of the tissue-specificity of N-glycosylation, their glycoforms may be different from those in serum. To date, no complete primary monosaccharide sequence of either the complex-type or the hybrid-type structures present in CNS tissue has been reported. Preliminary data on the glycoproteins proneurovasopressin, N-CAM, L1, J1, MAG, and Thy-1, all suggest "brain-specific" oligosaccharide structures (unpublished data). A comparative analysis of the class of the N-linked

oligosaccharides at each of the three N-glycosylation sites of mouse and rat brain Thy-1 suggests a close similarity (unpublished data), despite differences in the primary amino acid structure, implying a degree of conservation of neural N-glycosylation between species.

THE ROLE OF OLIGOSACCHARIDES IN THE ACTION OF GONADOTROPIC HORMONES

It is now well established that deglycosylation of human chorionic gonadotropin (hCG), either chemically or enzymatically, leads to an increased affinity of its binding to the receptor, but almost total loss of bioactivity (i.e. adenyl cyclase activation) (see 266 for references).

The glycohormone hCG is derived from the syncytiotrophoblast cells of the placenta, and is composed of two subunits, α and β, and is about 30% carbohydrate by weight (267). Both subunits contain two N-linked oligosaccharide chains, but additionally the β-subunit contains four O-linked chains. The structural analysis of the oligosaccharides (234, 235) has revealed that one of the structures on the α-subunit is a complex-type monoantennary chain, a structure not found to occur widely on other glycoproteins. On the basis of the molar proportions of the oligosaccharides present, it was proposed (234) that there is site-specific glycosylation, with each site being homogenous. This "lack" of heterogeneity, in relation to that often found for carbohydrate structures, may indicate a unique role for the oligosaccharides in the action of this hormone.

Treatment of hCG with neuraminidase leads to its rapid removal from the circulation (268), but treatment with neuraminidase and β-galactosidase has no significant effect on the half-life, leaves the receptor binding unaffected, but dramatically reduces adenyl cyclase activation (by ~85%) (268). A study of hCG in which either the α- or the β-subunit had been deglycosylated showed that only the oligosaccharides of the α-subunit were needed for coupling receptor binding to adenyl cyclase activation (269). Deglycosylated hCG was a potent competitive inhibitor of the binding of the native hormone (269). In contrast, glycopeptides, or free oligosaccharides, derived from the hCG α-subunit, inhibited in an uncompetitive way the adenyl cyclase activation, but did not inhibit receptor binding of hCG (266). This inhibition was specific, in that complex N-linked oligosaccharides from several other glycoproteins showed no such inhibition (266).

Supporting evidence for the role of the oligosaccharides comes from the properties of hCG produced in patients with choriocarcinoma (236). This hCG had the same amino acid composition as normal hCG and also contained

four N-linked oligosaccharides. However, the structures of its sugar chains were quite different from those on the "native" hormone, due to an increase in fucosyl transferase activity and the novel expression of an abnormal β-N-acetylglucosaminyl transferase IV in the choriocarcinoma cells. This hCG was found to have a much lower biological activity, but a threefold increase in affinity for its receptor when compared with native hCG (270). These results are consistent with an increase in receptor-binding affinity and a reduction in biological activity for the deglycosylated hCG.

These data suggest that the N-linked oligosaccharides of the α-subunit of hCG play a crucial part in signal transduction during a presumably lectin-mediated event occurring after binding of hormone to the receptor, and before activation of adenyl cyclase. Two recent observations suggest that, while such an idea is probably essentially correct, the actual details are more complex. First, it has been reported that there exist two "types" of receptors for hCG, on different cell types (271, 272). One of these (common on the light cell-fraction of Leydig cells) binds hCG tightly, but there is no subsequent steroidogenic activity. The other receptor (found on the heavy cell-fraction of Leydig cells) responds to hCG with increased steroidogenesis, but no binding by these cells of hCG can be detected. It was implicit in previous studies comparing effects of glycosylation on binding and biological activity that a single receptor existed. Second, the biological inactivity of the deglycosylated hCG can be reversed by binding certain antibodies (or their Fab fragments) directed only against the β-subunit (273). This observation is not easily reconciled with the above results (266). It is possible that the N-linked oligosaccharides of the anti-β subunit IgG acted in a "surrogate" fashion to mimic the missing oligosaccharides of the β-subunit. Alternatively, if the α-subunit oligosaccharides interact with the β-subunit, to convert the β-subunit into a configuration in which the β-subunit can cause adenyl cyclase activation, then certain antibodies against the β-subunit may restore biological activity. Support for this idea comes from the analysis of the free α-subunits present in the urine of healthy pregnant women or patients with tumors. In all cases, no monoantennary oligosaccharides, which normally occur on α-subunits present in the bioactive $\alpha\beta$ complexes, were found on the free α-subunits. None of the free α-subunits were able to combine with β-subunits, suggesting to the authors that the sugar may play an important role in the assembly of the complex (237). However, as discussed above, deglycosylated α-subunits can associate with β-subunits to produce a recombinant hCG that is a potent competitive inhibitor of native hCG. A resolution of these apparent discrepancies requires an identification of the putative receptor for the oligosaccharides on the α-subunit.

This coupling of receptor occupation to adenyl-cyclase activation by the

oligosaccharides of the α-subunits is equally important to the mode of action of other gonadotropic hormones [luteinizing hormone (LH), follicle-stimulating hormone (FSH)], and thyroid-stimulating hormone (TSH). It has been convincingly shown for all these hormones that the presence and integrity of the N-linked oligosaccharides of the α-subunit are essential for the activation of adenyl cyclase in the target tissue—but not for binding to the hormone receptor (274–276). The N-linked oligosaccharides of the β-subunit do not appear to be involved in either event. Recombinant glycohormones (of LH and FSH), in which either the α or the β subunit, or both, had been chemically deglycosylated, were studied for receptor binding and cAMP production (274). Deglycosylation was consistently associated with increased affinity of binding to the receptor. Deglycosylation of just the β-subunit had little effect on cAMP production, but deglycosylation of the α-subunit virtually abolished this. Further, the deglycosylated glycohormones were potent competitive inhibitors of binding of the native hormone. The recent discovery of naturally occurring FSH antagonists that block the biological activity of FSH but that show virtually identical immunoreactivity to FSH raises the possibility that deglycosylated (or atypically glycosylated) forms of the FSH polypeptide may be naturally used as FSH antagonists (276a).

MHC AND GLYCOSYLATION

Many differentiation antigens, some carbohydrate in nature, are transiently expressed and tolerated without any apparent autoimmune response. The mechanism(s) for this tolerance have not yet been fully defined. It has been proposed that cross-reactivity between the protein determinants of self antigens and self MHC antigens (particularly Class II) could prevent an autoimmune reaction (277). An alternative hypothesis is that potential autoantigens (for example, the proteolytic cleavage products of the complement system) are closely linked genetically to particular MHC antigens that are able to create an immune repertoire tolerant of those autoantigens (277). Clearly, since not all differentiation antigens are encoded within the MHC, alternative mechanisms are necessary for the induction of tolerance to differentiation antigens. Cross-reactivity between differentiation antigens and MHC antigens, without encoding for them within the MHC, could be possible if certain structural elements of the differentiation antigens were determined by loci within the MHC. An obvious possibility is that glycosylation of cell-surface antigens is under the influence of the MHC, and this has been suggested (278, 279).

The weight of current data now suggests that transfected class I antigens can still be recognized after deletion of the N-glycosylation sites (280). This

result firmly establishes that oligosaccharides residues on the class I MHC antigens themselves are not important for the recognition of these molecules. However, evidence does exist to suggest that the MHC loci can affect glycosylation. For example, Ia antigenic specificities can be either protein or carbohydrate (281). Further, certain H-2 mutant mice simultaneously express both altered protein and altered carbohydrate antigens (282). Glycopeptides obtained from various murine tumor cell lines have also been found to strongly inhibit the binding of primed allospecific cytotoxic T-cells to their target in an H-2-restricted manner (283, 284) (i.e. the glycopeptide extracts must be derived from a cell line expressing the same H-2 haplotype as the target cells). In this study (284), the authors concluded that "cytotoxic lymphocytes recognise, in part, carbohydrate structures on the surface of target cells whose specific structure is linked to, or determined by, genes of the MHC." To date, two enzymes affecting glycosylation have been mapped to the MHC. The loci controlling the activities of neuraminidase in liver (285) and activated T-cells (286) map within the MHC, as do those of the cell-surface galactosyl transferase (86), and the GM_1 synthetase [UDP-galactose:GM_2(NeuGc) galactosyl transferase] (287). Collectively, these results provide experimental support for the idea that MHC loci can exert an influence on the glycosylation capacity of a cell. If true, this would have profound implications for our understanding of the immunobiology of the MHC. Proof requires the demonstration of haplotype-specific glycosylation patterns. In addition, the direct involvement of N-linked oligosaccharides as immunoregulatory agents has recently been demonstrated (288, 289). The Tamm-Horsefall glycoprotein (uromodulin) isolated from human pregnancy urine (85 kd), containing 30% carbohydrate, is thought to suppress T-cell proliferation primarily by binding to, and lowering the activity of, the T-cell growth factor, interleukin-1. It has been shown that the uromodulin N-linked oligosaccharides alone are immunosuppressive and that the binding of uromodulin to interleukin-1 (K_d 3 × 10^{-10}), involves the oligosaccharides, thereby also establishing lectinlike properties for interleukin-1 (289). With the recent finding that TNF also binds to uromodulin it would seem fruitful to explore the extent to which oligosaccharide-lectin interactions are used to regulate the circulating levels of the lymphokines (296).

ACKNOWLEDGMENTS

We thank Professor Nathan Sharon for his critical reading of the manuscript and his comments and suggestions. We also thank Dr. S. Perkins for help with Figures 1–3. The Oligosaccharide Group is supported by the Monsanto Co.

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EXHIBIT B-4

ENVIRONMENTAL EFFECTS ON PROTEIN GLYCOSYLATION

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Cultured mammalian cells are being used to produce proteins for therapeutic and diagnostic use because of their ability to perform complex post-translational modifications, including glycosylation. The oligosaccharide moieties can play an important role in defining several biological properties of glycoproteins, including clearance rate, immunogenicity, and biological specific activity. There is a growing interest in defining the factors that influence glycosylation, including the cell culture environment. In this review we organize the published data from *in vitro* cell culture and tissue culture studies that demonstrate direct effects of the culture environment on N-linked glycosylation.

The majority of extracellular proteins of higher animals, including many proteins of potential pharmacological importance, are glycoproteins. The oligosaccharide side-chains are covalently attached to asparagine, threonine or serine side chains on the protein backbone—that is, they are either "N-linked" or "O-linked" (reviewed in refs. 1 and 2). The sequence of oligosaccharide processing reactions for N-linked glycosylation begins with the synthesis of a lipid-linked, oligosaccharide moiety ($Glc_3Man_9GlcNAc_2$-P-P-dol) and its transfer *en bloc* to the nascent polypeptide chain in the endoplasmic reticulum (ER) (Fig. 1, reaction 1). A series of "trimming" reactions are catalyzed by exoglycosidases in the ER, leading to a variety of "high mannose" oligosaccharide structures as the proteins exit (Fig. 1, reactions 2, 3 and 4). Oligosaccharide processing optionally continues in the compartments of the Golgi, with a variety of exoglycosidase and glycosyltransferase reactions leading to the generation of "complex type" oligosaccharide structures (Fig. 1, reactions 5 through 11)[2-5].

A given glycoprotein may have one or many sites for the attachment of oligosaccharide moieties. For example, IgG usually contains just one N-linked oligosaccharide chain in the constant heavy chain region, with the oligosaccharide moiety constituting less than 5% by weight of the glycoprotein[6]. Murine or human IgM typically has five N-linked oligosaccharide moieties in the constitutive heavy chain region[7], representing about 10% by weight of the glycoprotein. The oligosaccharide moieties of erythropoietin represent approximately 40% by weight of this glycoprotein[8].

Given the location of these carbohydrate groups on the outer surface of the protein, it is not surprising that the oligosaccharide moiety can have a significant effect on the physical/chemical properties of the protein, including thermal stability and solubility[9]. The oligosaccharide moieties also affect the "biological" properties of the glycoprotein. For example, it has been demonstrated *in vivo* that oligosaccharides (particularly terminal sialic acid groups) play an important role in defining the immunogenicity of a glycoprotein (reviewed in refs. 10 and 11). Protein clearance from the circulatory system is primarily mediated by recognition of specific oligosaccharide moieties (reviewed in refs. 12–14; see also refs. 15 and 16). Therefore, differences in oligosaccharide structure can dramatically affect the clearance rate of an injected glycoprotein[15].

In addition to their effect on clearance rate, the oligosaccharide moieties sometimes play a significant role in determining the "biological specific activity" of glycoproteins—that is, the activity per gram of glycoprotein (reviewed in ref. 17). For example, glycosylation can have a significant effect on the biological specific activity of several pituitary and placental glycoprotein hormones toward their target cells (reviewed in refs. 18 and 19); complete deglycosylation of thyrotropin results in reduced biological specific activity toward the target cells in spite of normal or enhanced receptor-binding characteristics. In fact, the deglycosylated hormones serve as competitive inhibitors of the native, glycosylated form[20,21].

An example of the control of *in vivo* biological activity through modulation of glycosylation comes from studies of IgE potentiating factor (IgE-pF) and IgE suppressive factor (IgE-sF), proteins capable of either stimulating or inhibiting the synthesis of IgE by lymphocytes. Recent evidence suggests that these glycoproteins may have the same protein core structure, and that their biological activity is determined by their respective oligosaccharide moieties (reviewed in refs. 22 and 23). Treatment of IgE-pF with neuraminidase results in loss of biological specific activity, suggesting that terminal sialic acid residues are necessary for IgE-pF potentiating activity[24].

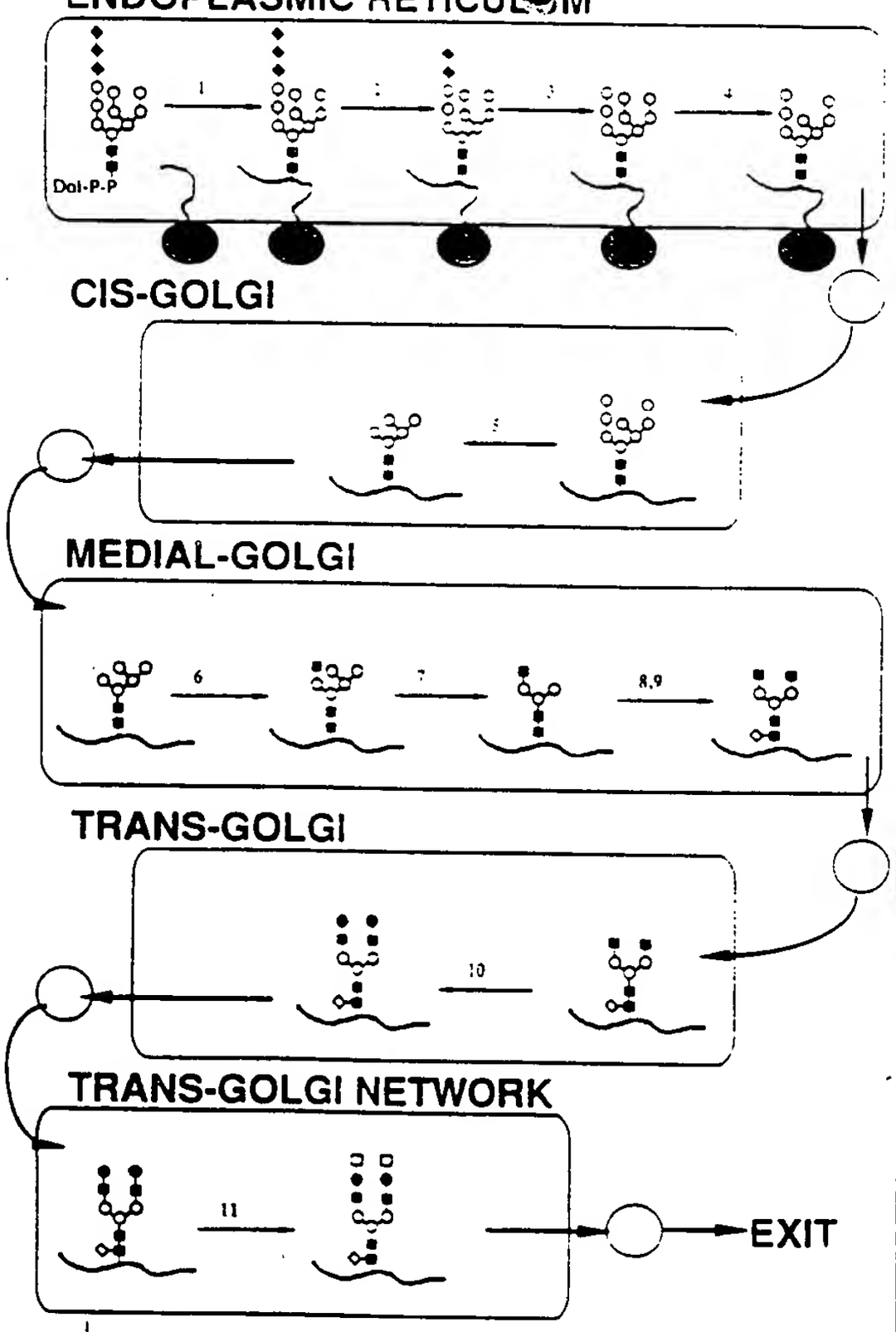

FIGURE 1 Pathway of oligosaccharide processing. The enzymes are: (1) oligosaccharyltransferase, (2) α-glucosidase I, (3) α-glucosidase II, (4) ER α-1,2-mannosidase, (5) Golgi α-mannosidase I, (6) N-acetylglucosaminyltransferase I, (7) Golgi α-mannosidase II, (8) N-acetylglucosaminyltransferase II, (9) fucosyltransferase, (10) galactosyltransferase, (11) sialyltransferase. The symbols are: ■, N-acetylglucosamine (GlcNAc); ○, mannose (Man); ◆, glucose (Glu); ◇, fucose (Fuc); ●, galactose (Gal); □, sialic acid (NANA). Dol-P-P is Dolichyldiphosphoryl. (Derived from figure 3 of Kornfeld and Kornfeld, 1985[2]).

Oligosaccharide moieties also affect the biological specific activity of immunoglobulins. For example, the effectiveness of Fc receptor binding and complement activation by IgG and IgM are dependent on glycosylation in the heavy chain[25–31]. Wallick and coworkers[32] have recently demonstrated that glycosylation in the IgG variable region can affect antibody affinity for antigen. Aberrant glycosylation does not prevent secretion of IgG and IgM[33].

Other examples where glycosylation has been implicated in modulating biological specific activity (apart from effects on clearance rate) include: t-PA[34,35], prolactin[36,37], fibrinogen[38], erythropoietin (EPO)[38,39], human granulocyte/macrophage colony-stimulating factor[40], epidermal growth factor receptor[41], fibroblast growth factor receptor[42], transferrin receptor[43] and fibronectin[44].

Cultured mammalian cells are being used to produce proteins for therapeutic and diagnostic use because of their ability to perform complex post-translational modifications, including glycosylation. In this context, there has been growing interest in defining the factors that influence oligosaccharide structure. It is now well-established that glycosylation is species-specific[2,17]. Glycosylation can also be tissue- and cell type-specific within a given species[17,45,46]. Differences in glycosylation have been observed between proteins synthesized by normal and highly-transformed mammalian cells (reviewed in refs. 1 and 47, see also refs. 48–55). These factors assure that a particular recombinant protein will have host-dependent oligosaccharide structures[8,56–58], which may (or may not) affect its *in vivo* clearance rate and biological specific activity.

Two lines of evidence suggest that the extracellular environment may affect glycosylation. First, significant *in vivo* changes in glycosylation are observed in association with a number of physiological states[59,60] including pregnancy[61–64]. This review will focus on the second line of evidence—*in vitro* cell culture or tissue culture studies that have demonstrated direct effects of culture environment on N-linked glycosylation.

ENVIRONMENTAL EFFECTS ON GLYCOSYLATION

Glucose starvation. Glucose starvation leads to two distinct abnormalities in the synthesis of glycoproteins: (1) attachment of aberrant precursor oligosaccharides and (2) the absence of oligosaccharide moieties at asparaginyl sites, which are normally glycosylated (reviewed in ref. 65). Under normal conditions, N-linked glycosylation commences cotranslationally with the addition of the species $Glc_3Man_9GlcNAc_2$ to the growing polypeptide chain, in conjunction with its entry into the endoplasmic reticulum. The attachment of smaller precursor oligosaccharides, such as $Glc_3Man_5GlcNAc_2$ has been observed in glucose-starved Chinese hamster ovary cells[66,67], mouse 3T3 cells[68], rat hepatoma cells[69] and normal rat kidney cells[70].

The second type of defect in oligosaccharide processing was first observed in the production of immunoglobulin light chain by a glucose-starved mouse myeloma (MOPC-46)[71]. Glucose starvation resulted in the absence of oligosaccharide moieties at light chain asparaginyl sites that are normally glycosylated. The effect was concentration-dependent up to 80 mg/liter of glucose. This phenomenon has also been observed in glucose-starved CHO cells[67].

An interesting observation from these studies is that the inhibition of oligosaccharide processing by glucose-starvation was relieved by mannose addition, but not by substitution of fructose, pyruvate, glutamine and a number of other carbohydrates[68,71,72].

In contrast to the substantial effects of glucose starvation noted above, Ronin and coworkers noted relatively minor effects of glucose-starvation on oligosaccharide processing for thyroid tumor cells[73].

Hormonal effects. Many *in vitro* studies have documented the role of hormones in regulating the oligosaccharide structure of glycoproteins. For example, thyrotropin-releasing hormone (TRH), a tripeptide secreted by hypothalamic neurons, stimulates pituitary synthesis of the glycoprotein thyrotropin; recent evidence suggests

motes alteration of thyrotropin oligo-
es with a resulting increase in thyrotro-
[20,74,75]. Thyrotropin, in turn, stimu-
thyroid cells of thyroglobulin (Tg), a
rsor of thyroid hormones; thyrotropin
osaccharide structure of Tg, increasing
no- and disialvated derivatives of the
ains[76]. The TRH/thyrotropin/Tg exam-
ustrates the potential for changes in
plify a cascade of events associated with
ion of cell metabolism.

mples of hormonal effects on glycosvia-
ted. Regulation of IgE binding factor
ructure has also been observed to be
external protein factors[28,77]. Dexameth-
monstrated to affect glycoprotein oligo-
re in rat hepatocytes[78] and a rat hepato-
gonadotropin-releasing hormone regu-
synthesis and glycosylation in cultured
wo additional examples of hormonal
lation include thyroid hormone trilo-
regulation of α-lactalbumin glycosvla-
mmary gland explants[82], and prolactin
sferrin glycosylation in rabbit mammary

portant *in vivo* roles of hormones is
rentiated phenotype. For example, reti-
nd its metabolic product retinoic acid
portant *in vivo* role in epithelial cell
Exposure of cultured mammalian cells
oic acid results in a variety of cell type-
s[84], including changes in glycoprotein
rocessing. Bernard and coworkers[85] ob-
sure of chondrocytes to retinoic acid
ion of fibronectin from high mannose-
ype. Lotan and coworkers[86,87] observed
ration of sialic acid, galactose, and N-
into a specific mouse melanoma sur-
sponse to retinoid acid treatment. Such
rently associated with the known func-
he induction of differentiated pheno-
of other synthetic agents used routine-
the differentiated state have also been
glycosylation, including DMSO[90], and
s PMA[91,92] and TPA[93]. The effects vary
cell type and inducing agent, probably
e-specific effects of each of these agents

cy alters protein glycosylation *in vivo* by
parently distinct from that discussed
nt study employing isolated liver micro-
s suggests that this effect may be due to
initial protein glycosylation reaction[96]
. 1). This result suggests the possibility
tein glycosylation in vitamin A-deficient
f the "underglycosylation" mechanism
for glucose starvation[56].

nines. Weak bases, a category composed
of amines, have disruptive effects on
rocessing. For example, addition of 10
ltured plasma cells results in the secre-
ient in terminal sialylation[97]. Tris buffer
e, dependent upon an amine group for
bility. At 20 mM, Tris inhibits sialylation
patocytes, and additionally inhibits proc-
drates from high-mannose to complex

mental agents. Several reports have indi-
effects of environment on the glycosyla-
tracellular proteins in contexts that are

significant to biotechnology. For example, Anderson and coworkers[99] noted differences in IgM sialylation from different preparations of ascites fluid. They further noted that IgM produced in cell culture exhibited incomplete oligosaccharide processing in comparison to IgM produced in ascites fluid. In contrast, Moellering and coworkers[100] observed no difference in oligosaccharide structure for immunoglobulin synthesized in ascites fluid or in serum-free or serum-supplemented cell culture.

A variety of other environmental components have been reported to alter oligosaccharide processing of glycoproteins, including hydrogen ion concentration[101,102], platelet-derived growth factor[103,104], EDTA[105] and HEPES buffer[101,106]. Additional analyses are necessary to confirm and extend these results.

Finally, glycoprotein oligosaccharide structure is affected by a variety of agents not normally found in cell culture medium (reviewed in ref. 65). Notable members of this group include: (1) tunicamycin, an antibiotic that blocks N-linked glycosylation by disrupting the assembly of the precursor oligosaccharide, and (2) monensin, an ionophore that disrupts movement of glycoproteins between intracellular compartments, and therefore disrupts Golgi processing of oligosaccharides to complex form.

MECHANISMS FOR ALTERATION OF OLIGOSACCHARIDE PROCESSING

Inhibition of oligosaccharide precursor assembly and transfer to peptide. As mentioned previously, one potential consequence of glucose-starvation is attachment of an abbreviated oligosaccharide precursor to the nascent peptide. This effect could be related to both the energy depleted state of the cell and the shortage of glucose-derived oligosaccharide precursors. In fact, the effect can be mimicked by exposure of cells to carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)[107], an uncoupler of oxidative phosphorylation. Since glucose transport into the cell is energy dependent, it is possible that the effect of CCCP results in glucose deficiency within the cell.

A second consequence of glucose starvation is the absence of oligosaccharide at a asparaginyl site that is usually glycosylated. Such an effect can be expected under conditions leading to a shortage of the oligosaccharide precursor. This effect is clearly evident in experiments involving the antibiotic tunicamycin, which blocks synthesis of oligosaccharide precursor. The resulting protein is imported into the endoplasmic reticulum minus any carbohydrate moiety. If the lack of oligosaccharide results in improper folding or aggregation, the polypeptide may be trapped in the endoplasmic reticulum, bound to BIP[108]. In some cases, though, the protein is secreted absent any carbohydrate structure. Such is the case with IgG, which is secreted in the presence of tunicamycin without carbohydrate, with subsequent reduction in biological specific activity[27].

Genetic modulation of exoglycosidase and glycosyltransferase activities. Glycoproteins are the substrates for oligosaccharyltransferase (Fig. 1, reaction 1) and for the variety of exoglycosidase and glycosyltransferase enzymes in the endoplasmic reticulum and Golgi (Fig. 1). The activities of these enzymes will be regulated by the variety of mechanisms associated with the regulation of other cellular enzymes (e.g. enzyme concentration, etc.). For example, stimulation of thyroid cells by thyroglobulin leads to increased per cell activity of several glycosyltransferases, including oligosaccharyltransferase[109,110]. Up- and down-regulation of specific glycosyltransferases has been observed frequently in conjunction with hormonal induction of differentiated phenotype[87,90,91,93,111,112]. For example, Durham and coworkers[93] observed increased

activity of specific galactvl-, fucosvl-, and sialvltransferases and decreased activity of a fucosvltransferase in mouse myeloid (HL60) cells treated with retinoic acid.

Many of the hormones affecting glycosylation are associated with well-characterized mechanisms for transcriptional regulation of gene expression (e.g. dexamethasone and retinoic acid[113,114]). Presumably, transcriptional control of glycosylation enzyme concentrations is responsible for many of the effects on oligosaccharide processing associated with hormones. Wang and coworkers[115] have recently confirmed this hypothesis using hepatocytes and a hepatoma cell line: exposure to dexamethasone resulted in a three- to four-fold increase in sialyltransferase activity due to a corresponding increase in sialyltransferase mRNA through a transcriptional control mechanism.

Interference with ER/Golgi environment. Most of the enzymes involved in the biosynthetic route outlined in Figure 1 have been characterized to some extent, and these studies suggest regulation of oligosaccharide processing through direct effects at the enzyme level. In general, glycosylation enzymes have pH optima that range between neutral and acidic[116–122]. Several enzymes have stringent divalent cation requirements and are completely inhibited by EDTA treatment[117]; the preferred cations are typically Mn^{+2}[116,123–125] and Ca^{+2}[117,125]. Many of the oligosaccharide processing enzymes exist in multimeric complexes in their native state[118,125–127]. Several of these enzymes have been identified as glycoproteins[117,118,120,121].

Agents that interfere with the environment within ER or Golgi compartments, such as amines (weak bases), can be expected to affect oligosaccharide processing. The disruptive mechanism of amines is understood in a general sense (reviewed in ref. 128). Amines in their neutral form diffuse through the cell membrane or in their charged form may be carried into the cell by surface transporters. Inside the cell they tend to accumulate in pH-sensitive, acidic intracellular compartments to concentrations in excess of their extracellular concentration. As a result, the pH of these compartments is raised, resulting in inhibition of pH-sensitive enzyme activity and disruption of receptor-ligand interactions[128,129]. The interference by ammonium ion and Tris with late oligosaccharide processing events is apparently related to their accumulation in an acidic intracellular compartment in the Golgi[97,98,130–132].

Interference with vesicle trafficking. The glycosylation pathway is dependent upon vesicle transport between the ER and subsequent Golgi compartments. Some glycoproteins are also subject to sorting mechanisms that regulate their direction into regulated or constitutive secretion pathways (reviewed in refs. 133, 134). Environmental agents can disrupt vesicle transport and sorting with subsequent effects on glycosylation. For example, amines interfere with the intracellular membrane fusions necessary for vesicle trafficking within the cell[128]. These transport and sorting steps are also very sensitive to intracellular ion concentrations, and agents that interfere with intracellular ion concentrations disrupt oligosaccharide processing. Treatment of cells with the ionophore monensin disrupts transport from the ER to the Golgi, leading in some cases to secretion of glycoproteins which have been only partially processed[135]. Hormonal regulation of vesicle trafficking is also possible: Haffar and coworkers[60] have shown that glucocorticoids regulate the intercellular trafficking of MMTV glycoproteins synthesized in rat hepatoma cells.

POSTSCRIPT

Protein translation occurs on the basis of a mRNA template, assuring high fidelity of protein structure. In contrast, oligosaccharide processing occurs as a result of the sequential actions of several enzymes in different intracellular compartments. It should not be surprising that the outcome of this set of reactions varies with environmental conditions. *In vivo*, this variability apparently serves an important function within the cell, permitting fine regulation of the clearance rate and biological activity for at least some proteins. *In vitro*, potential environmental affects on oligosaccharide structure cannot be ignored, given the importance of oligosaccharide structure in defining clearance rate, biological specific activity and immunogenicity.

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different paths, and by ensuring that an azimuthally uniform coverage of stations is used in the averaging calculation. To compensate for other factors, such as focal depth, fault geometry and corner frequency would require such a detailed knowledge of the earthquake source that the M_s measurement itself would be redundant.

The results of this analysis can be summarized in five points.

1) A global average moment-magnitude relationship M_s has been defined which can be used to predict M_s over a wide range of magnitudes and scalar moments.

2) The variance of surface wave measurements for an event of a particular scalar moment is ~0.2 magnitude units.

3) Large regional biases in M_s exist.

4) Differences in source scaling may explain some of the differences. Specifically, observations show that the transition from a slope of unity to a smaller value occurs at large moments for continental events than for ridge and fracture zone events, suggesting systematic differences in stress drop.

5) Other systematic factors affecting the calculation of M_s also appear to contribute to the observed regional bias.

We thank Professor J. H. Woodhouse for reading and correcting the manuscript and Professor H. Kanamori for constructive criticism throughout our work on this subject. This work was supported by the NSF.

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Reshaping human antibodies for therapy

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A human IgG1 antibody has been reshaped for serotherapy in humans by introducing the six hypervariable regions from the heavy- and light-chain variable domains of a rat antibody directed against human lymphocytes. The reshaped human antibody is as effective as the rat antibody in complement and is more effective in cell-mediated lysis of human lymphocytes.

IN 1890 it was shown that resistance to diphtheria toxin could be transferred from one animal to another by the transfer of serum. It was concluded that the immune serum contained an anti-toxin, later called an antibody[1]. For many years animal antisera were used in the treatment of microbial infections and for the neutralization of toxins in man[2]. More recently rodent monoclonal antibodies (mAbs)[3] have been used as 'magic bullets'[4] to kill and to image tumours[5,6]. The foreign immunoglobulin, however, can elicit an anti-globulin response which may interefere with therapy[7] or cause allergic or immune complex hypersensitivity[2]. Thus ideally human antibodies would be used. Human immunoglobulins are widely used as both prophylactic and microbicidal agents[3], but it would be far better to have available human mAbs of the desired specificity. It has proven difficult, however, to make such mAbs by the conventional route of immortalization of human antibody-producing cells[8].

There is an alternative approach. Antibody genes have been transfected into lymphoid cells, and the encoded antibodies expressed and secreted; by shuffling genomic exons, simple chimaeric antibodies with mouse variable regions and human constant regions have been made[9–12]. Such chimaeric antibodies have at least two advantages over mouse antibodies. First, the effector functions can be selected or tailored as desired. For example, of the human IgG isotypes, IgG1 and IgG3 appear to be the most effective for complement and cell-mediated lysis[13–15], and therefore for killing tumour cells. Second, the use of human rather than mouse isotypes should minimize the anti-globulin responses during therapy[16,17] by avoiding anti-isotypic antibodies. The extent to which anti-idiotypic responses to rodent antibodies in therapy are dictated by foreign components of the variable versus the constant region is not known, but the use of human isotypes should reduce the anti-idiotypic response. For example, when mice were made tolerant to rat immunoglobulin constant-region determinants, administration of rat anti-lymphocyte antibodies did evoke anti-idiotypic responses, but these were delayed and weaker than in animals that had not been made tolerant[15]. Nevertheless, it is likely that a chimaeric antibody would provoke a greater immune response than a human mAb.

We have attempted to build rodent antigen binding sites directly into human antibodies by transplanting only the antigen binding site, rather than the entire variable domain, from a rodent antibody. The antigen binding site is essentially encoded by the hypervariable loops at one end of the β-sheet framework. The hypervariable regions of the heavy chain of mouse antibodies against a hapten[18] or a protein antigen[19] were previously transplanted into a human heavy chain, and, in association with the mouse light chain, the antigen binding site was retained.

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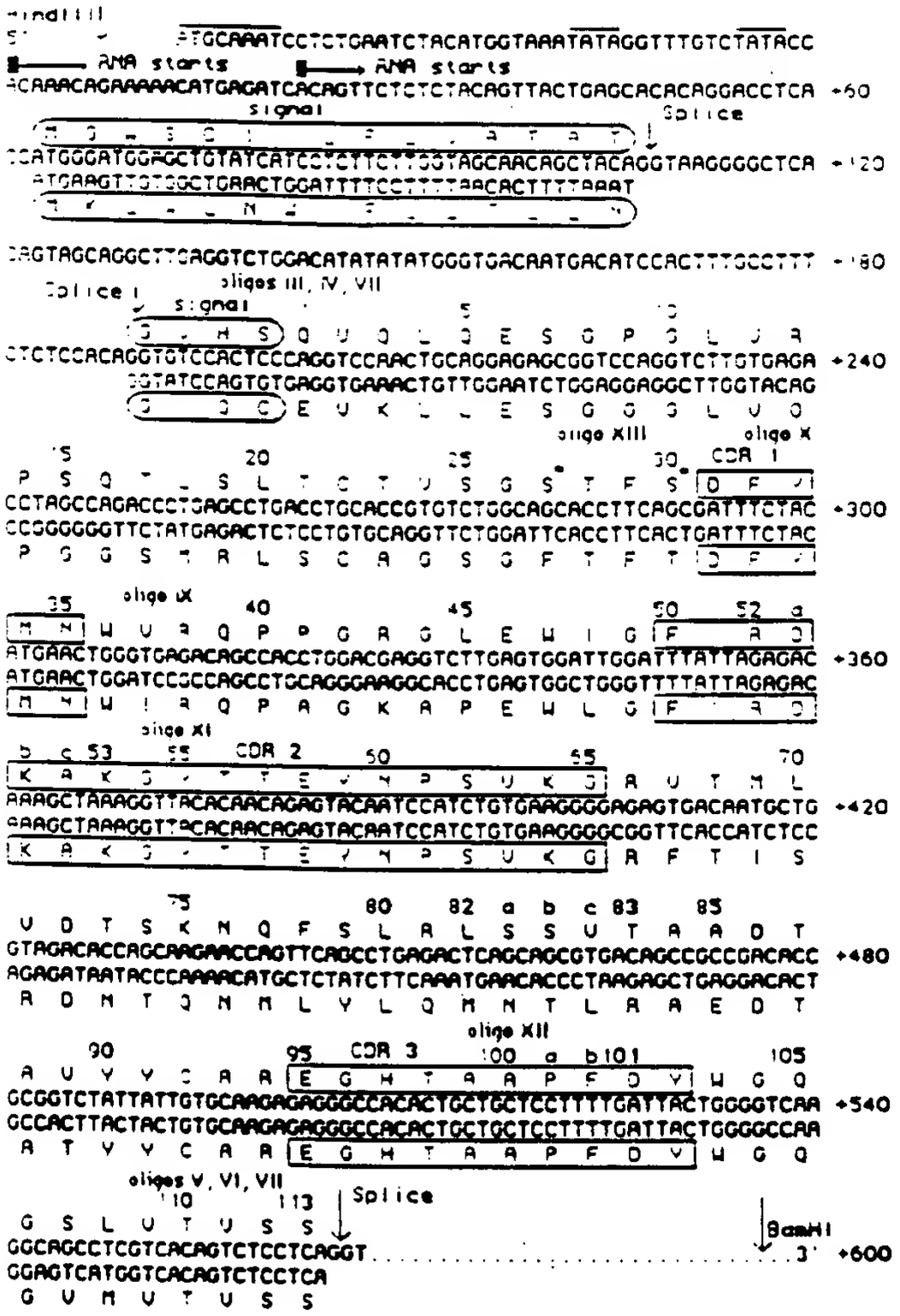

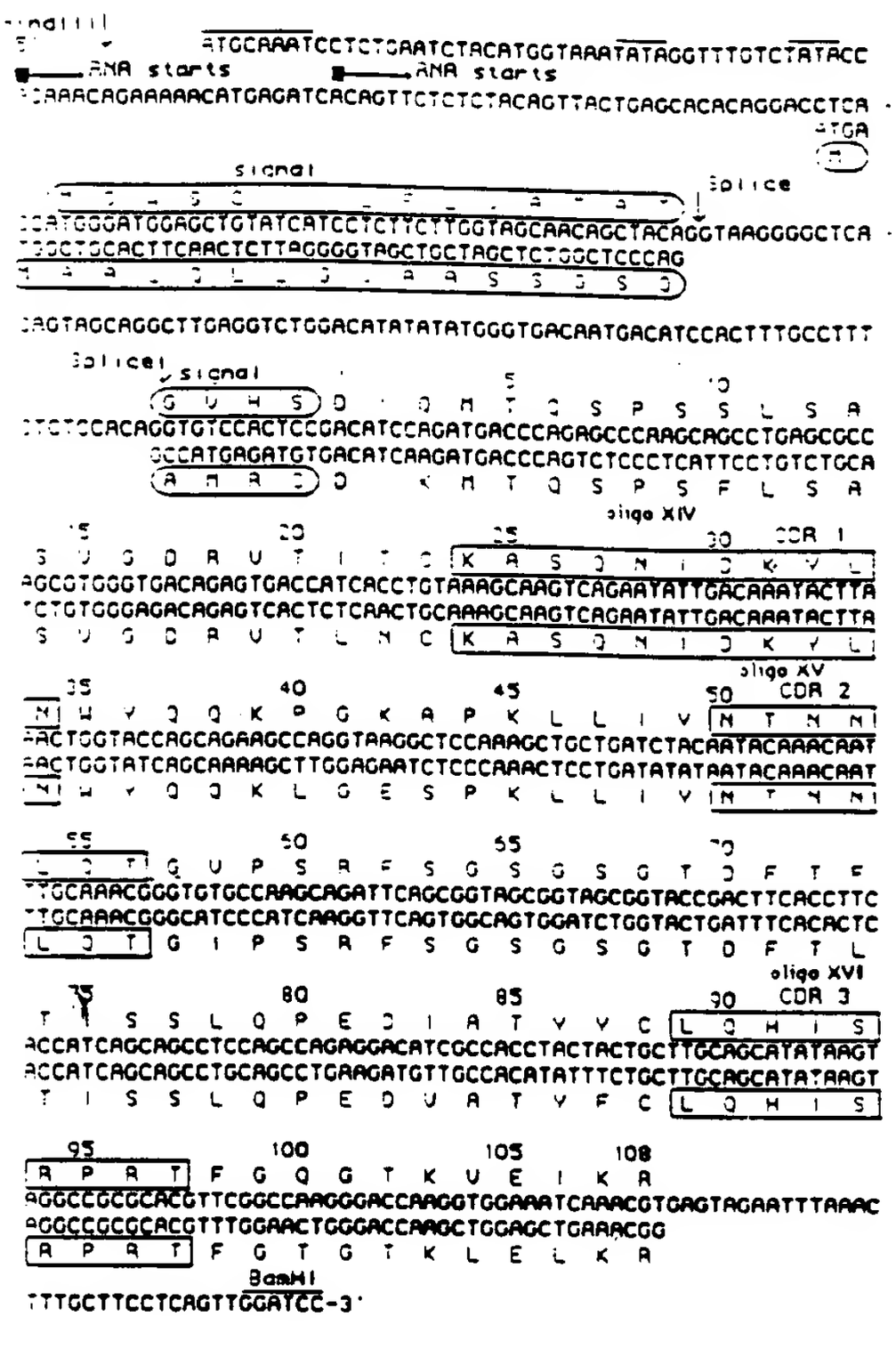

Fig. 1 Heavy-chain (*a*) and light-chain (*b*) sequences of the variable domains of reshaped (upper line) or rat YTH 34.5HL (lower lin antibodies. The reshaped heavy-chain variable domain HuVHCAMP was based on the HuVHNP gene[18,19], with the framework regions human NEW (see note) alternating with the hypervariable regions of rat YTH 34.5HL. The reshaped light-chain variable domain HuVLCAM is a similar construct, except with the framework regions of the human myeloma protein REI, with the C-terminal and the 3' non-codi sequence taken from a human J_κ-region sequence[36]. The sequences of oligonucleotide primers are given and their locations on the genes a marked.

Methods. Messenger mRNA was purified[37] from the hybridoma clone YTH 34.5HL (γ2a, κ^b). First strand cDNA was synthesized by primi with oligonucleotides complementary to the 5' end of the CH1 (oligonucleotide I) and the Cκ exons (oligonucleotide II), and then clon and sequenced as described previously[38,39]. Two restriction sites (*Xba*I and *Sal*I) were introduced at each end of the rat heavy-chain variab region RaVHCAMP cDNA clone in M13 using mutagenic oligonucleotides III and V respectively, and the *Xba*I–*Sal*I fragment was excise The corresponding sites were introduced into the M13-HuVHNP gene using oligonucleotides IV and VI, and the region between the sit was then exchanged. The sequence at the junctions was corrected with oligonucleotides VII and VIII, and an internal *Bam*HI site remov using the oligonucleotide IX, to create the M13-RaVHCAMP gene. The encoded sequence of the mature domain is thus identical to that YTH 34.5HL. The reshaped heavy-chain variable domain (HuVHCAMP) was constructed in an M13 vector by priming with three lo oligonucleotides simultaneously on the single strand containing the M13-HuVHNP gene[18,19]. Each oligonucleotide (X, XI and XII) w designed to replace each of the hypervariable regions with the corresponding region from the heavy chain of the YTH 34.5HL antiboc Colony blots were probed initially with the oligonucleotide X and hybridization positives were sequenced: the overall yield of the triple muta was 5%. The (Ser27 → Phe) and (Ser27 → Phe, Ser30 → Thr) mutants of M13mp8-HuVHCAMP were made with the mixed oligonucleoti XIII. The reshaped light-chain variable domain (HuVLCAMP) was constructed in M13 from a gene with framework regions based on hum REI (J. Foote, unpublished data). As above, three long oligonucleotides (XIV, XV and XVI) were used to introduce the hypervariable regio of the YTH 34.5HL light chain.

Note: There are discrepancies involving the first framework region and the first hypervariable loop of the NEW heavy chain between t published sequence[17] used here and the sequence deposited in the Brookhaven data base (in parentheses): Ser27 (→ Thr), Thr28 (→ Ser) a Ser30 (→ Asp). Neither version is definitive (R. J. Poljak, personal communication) and the discrepancies do not affect our interpretatio

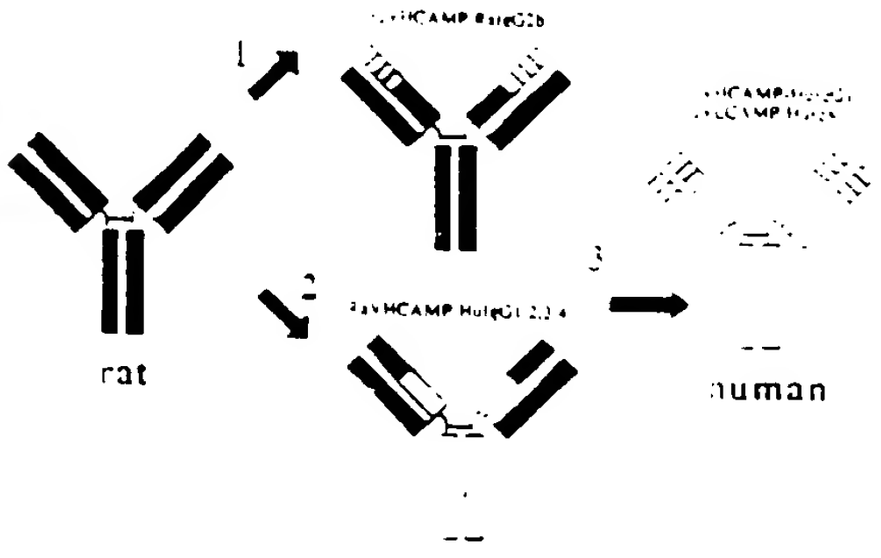

Fig. 2 Strategy for reshaping a human antibody for therapy. Sequences of rat origin are marked in black, and those of human origin in white. The recombinant heavy and light chains are also marked using a systematic nomenclature. See text for description of stages 1, 2 and 3. The genes encoding the variable domains were excised from the M13 vectors as *Hin*dIII-*Bam*HI fragments, and recloned into pSV2gpt[29] (heavy chains) or pSV2neo[30] (light chains), expression vectors containing the immunoglobulin enhancer[32]. The human γ1 (ref. 40), γ2 (ref. 41), γ3 (ref. 42), γ4 (ref. 41) and κ (ref. 36) and the rat γ2b (ref. 43) constant domains were introduced as *Bam*HI fragments. The following plasmids were constructed and transfected into lymphoid cell lines by electroporation[33]. In stage 1, the pSVgpt plasmids HuVHCAMP-RaIgG2B, HuVHCAMP(Ser→Phe)-RaIgG2B, HuVHCAMP-(Ser27→Phe, Ser30→Thr)-RaIgG2B were introduced into the heavy chain loss variant of YTH 34.5HL. In stage 2, the pSVgpt plasmids RaVHCAMP-RaIgG2B, RaVHCAMP-HuIgG1, RaVHCAMP-HuIgG2, RaVHCAMP-HuIgG3, RaVHCAMP-HuIgG4 were transfected as above. In stage 3, the pSV-gpt plasmid Hu(Ser27→Phe, Ser30→Thr)VHCAMP-HuIgG1 was co-transfected with the pSV-neo plasmid HuVLCAMP-HuIgK into the rat myeloma cell line Y0 (Y B2/3.0 Ag 20 (ref. 31). In each of the three stages, clones resistant to mycophenolic acid were selected and screened for antibody production by ELISA assays. Clones secreting antibody were subcloned by limiting dilution (for Y0) or the soft agar method (for the loss variant) and assayed again before 1 litre growth in roller bottles.

Since, to a first approximation, the sequences of hypervariable regions do not contain characteristic rodent or human motifs, such 'reshaped' antibodies should be indistinguishable in sequence from human antibodies.

There are mAbs to many cell-type-specific differentiation antigens, but only a few have therapeutic potential. Of particular interest is a group of rat mAbs directed against an antigen, the 'CAMPATH-1' antigen, which is strongly expressed on virtually all human lymphocytes and monocytes, but is absent from other blood cells including the haemopoietic stem cells[20]. The CAMPATH-1 series contains rat mAb of IgM, IgG2a and IgG2c isotypes[21], and more recently IgG1 and IgG2b isotypes which were isolated as class-switch variants from the IgG2a-secreting cell line YTH 34.5HL[22]. All of these antibodies, except the rat IgG2c isotype, are able to lyse human lymphocytes efficiently with human complement. Also the IgG2b antibody YTH 34.5HL-G2b, but not the other isotypes, is effective in antibody-dependent cell-mediated cytotoxicity (ADCC) with human effector cells[23]. These rat mAbs have important applications in problems of immunosuppression: for example control of graft-versus-host disease in bone-marrow transplantation[20]; the management of organ rejection[23]; the prevention of marrow rejection; and the treatment of various lymphoid malignancies (ref. 24 and M. J. Dyer, Hale, G., Hayhoe, F. G. J. and Waldmann, H., unpublished observations). The IgG2b antibody YTH 34.5HL-G2b seems to be the most effective at depleting lymphocytes *in vivo* but the use of all of these antibodies is limited by the anti-globulin response which can occur within two weeks of the initiation of treatment[25]. Here we describe the reshaping of human heavy and light chains towards binding the CAMPATH-1 antigen and the selection of human effector functions to match the lytic potential of the rat IgG2b isotype.

Table 1 Reshaping the heavy-chain variable domain

Heavy chain variable domain	Concentration of antibody in μg ml⁻¹ at 50% antigen binding	50% complement lysis
RaVHCAMP	0.7	2.1
HuVHCAMP	27.3	*
HuVHCAMP (Ser27→Phe)	1.8	16.3
HuVHCAMP (Ser 27→Phe, Ser 30→Thr)	2.0	17.6

Antibodies with the heavy-chain variable domains listed above, rat IgG2b constant domains and rat light chains were collected from supernatants of cells at stationary phase and concentrated by precipitation with ammonium sulphate, followed by ion exchange chromatography on a Pharmacia MonoQ column. The yields of antibody were measured by an enzyme-linked immunosorbent assay (ELISA) directed against the rat IgG2b isotype, and each was adjusted to the same concentration[35]. To measuring binding to antigen, partially purified CAMPATH-1 antigen was coated onto microtitre wells and bound antibody was detected via a biotin-labelled anti-rat IgG2b mAb[35], developed with a streptavidin-peroxidase conjugate (Amersham). Complement lysis of human lymphocytes was with human serum as the complement source[23]. For both binding and complement assays, antibody titres were determined by fitting the data to a sigmoid curve by at least squares iterative procedure[21].

* Complement lysis with the HuVHCAMP variable domain was too weak for the estimation of lytic titre.

Strategy

The amino-acid sequences of the heavy- and light-chain variable domains of the rat IgG2a CAMPATH-1 antibody YTH 34.5HL were determined from the cloned complementary DNA (Fig. 1), and the hypervariable regions were identified according to Kabat[25]. In the heavy-chain variable domain there is an unusual feature in the framework region. In most known heavy-chain sequences Pro41 and Leu45 are highly conserved: Pro41 helps turn a loop distant from the antigen binding site and Leu45 is in the β bulge which forms part of the conserved packing between heavy- and light-chain variable domains[26]. In YTH 34.5HL these residues are replaced by Ala41 and Pro45 and presumably this could have some effect on the packing of the heavy- and light-chain variable domains. Working at the level of the gene and using three large mutagenic oligonucleotides for each variable domain, the rat hypervariable regions were mounted in a single step on the human heavy- or light-chain framework regions taken from the crystallographically solved proteins NEW[27] and REI[28] respectively (Fig. 1). The REI light chain was used because there is a deletion at the beginning of the third framework region in NEW. The reshaped human heavy- and light-chain variable domains were then assembled with constant domains in three stage (Fig. 2). This permits a step-wise check on the reshaping of the heavy-chain variable domain (stage 1), the selection of the human isotype (stage 2), and the reshaping of the light-chain variable domain and the assembly of human antibody (stage 3). The plasmid constructions were genomic, with the sequences encoding variable domains cloned as *Hin*dIII-*Bam*HI fragments and those encoding the constant domains as *Bam*HI-*Bam*HI fragments in either pSVgpt (heavy chain)[29] or pSVneo (light chain)[30] vectors. The heavy-chain enhancer sequence was included on the 5′ side of the variable domain, and expression of both light and heavy chains was driven from the heavy-chain promoter and the heavy-chain signal sequence.

Heavy-chain variable domain

In stage 1, the reshaped heavy-chain variable domain (HuVHCAMP) was attached to constant domains of the rat

Fig. 3 Loop Phe27 to Tyr35 in the heavy-chain var domain of the human myeloma protein KOL, whicr been solved crystallographically[45]. The backbone o hypervariable region according to Kabat[25] is highlig and a 200% van der Waal surface is thrown around P to show the interactions with Tyr 32 and Met 34 o Kabat hypervariable region. In the rat YTH 34.5HL h chain, these three side chains are conserved in chai but in HuVHCAMP, Phe27 is replaced by Ser.

isotype IgG2b and transfected into a heavy-chain loss variant of the YTH 34.5 hybridoma. This variant carries two light chains, one derived from the Y3 fusion partner[31]. The cloned rat heavy-chain variable domain (RaVHCAMP) was also expressed as above, and the antibodies were purified and quantified (Table 1). The HuVHCAMP and RaVHCAMP antibodies, each of the rat IgG2b isotype, were compared to the CAMPATH-1 antigen in a direct binding assay and in complement lysis of human lymphocytes (Table 1). Compared with the original rat antibody, or the engineered equivalent, the antibody with the reshaped heavy-chain domain bound poorly to the CAMPATH-1 antigen and was weakly lytic. This suggested an error in the design of the reshaped domain.

There are several assumptions underlying the transfer of hypervariable loops from one antibody to another[47], in particular the assumption that the antigen binds mainly to the hypervariable regions. These are defined as regions of sequence[25] or structural[32] hypervariability, the locations of hypervariable regions being similar by both criteria except for the first hypervariable loop of the heavy chain. By sequence the first hypervariable loop extends from residues 31-35 (ref. 25) whereas by structure it extends from residues 26-32 (ref. 32). Residues 29 and 30 form part of the surface loop, and residue 27, which is phenylalanine or tyrosine in most sequences, including YTH 35.5HL, helps pack against residues 32 and 34 (Fig. 3). Unlike most human heavy chains, in NEW (see note in Fig. 1) the phenylalanine is replaced by serine, which would be unable to pack in the same way. To restore the packing of the loop, we made both a Ser 27 → Phe mutation, and a Ser 27 → Phe, Ser 30 → Thr double mutation in HuVHCAMP. These two mutants showed a significant increase in binding to CAMPATH-1 antigen and lysed human lymphocytes with human complement (Table 1). Thus the affinity of the reshaped antibody could be restored by a single Ser 27 → Phe mutation, possibly as a consequence of an altered packing between the hypervariable regions and the framework. This suggests that alterations in the 'Kabat' framework region can enhance the affinity of the antibody and extends previous work in which an engineered change in the hypervariable region yielded an antibody with increased affinity[33].

Heavy-chain constant domains

In stage 2 (Fig. 2), the rat heavy-chain variable domain was attached to constant domains of the human isotypes IgG1, 2, 3 and 4, and transfected into the heavy-chain loss variant of the YTH 34.5 hybridoma. In complement lysis (Fig. 4*a*), the human IgG1 isotype proved similar to the YTH 34.5HL-G2b, with the human IgG3 isotype being less effective. The human IgG2 isotype was only weakly lytic and the IgG4 isotype was non-lytic. In ADCC (Fig. 4*b*) the human IgG1 was more lytic than the YTH 34.5HL-G2b antibody. The decrease in lysis at higher concentrations of the rat IgG2b and the human IgG1 antibody is due to an excess of antibody, which causes the lysis of effector cells. The human IgG3 antibody was weakly lytic, and the IgG2 and IgG4 isotypes were non-lytic.

We therefore selected the human IgG1 isotype f reshaped antibody. Other recent work also favours the IgG1 isotype for therapeutic application. When the e functions of human isotypes were compared using a chimaeric antibodies with an anti-hapten variable doma IgG1 isotype appeared superior to the IgG3 in both comp and cell-mediated lysis[15]. Also, of two mouse chimaer bodies with human IgG1 or IgG3 isotypes directed aga surface antigens as tumour cell markers, only the IgG1 mediated complement lysis[13,14].

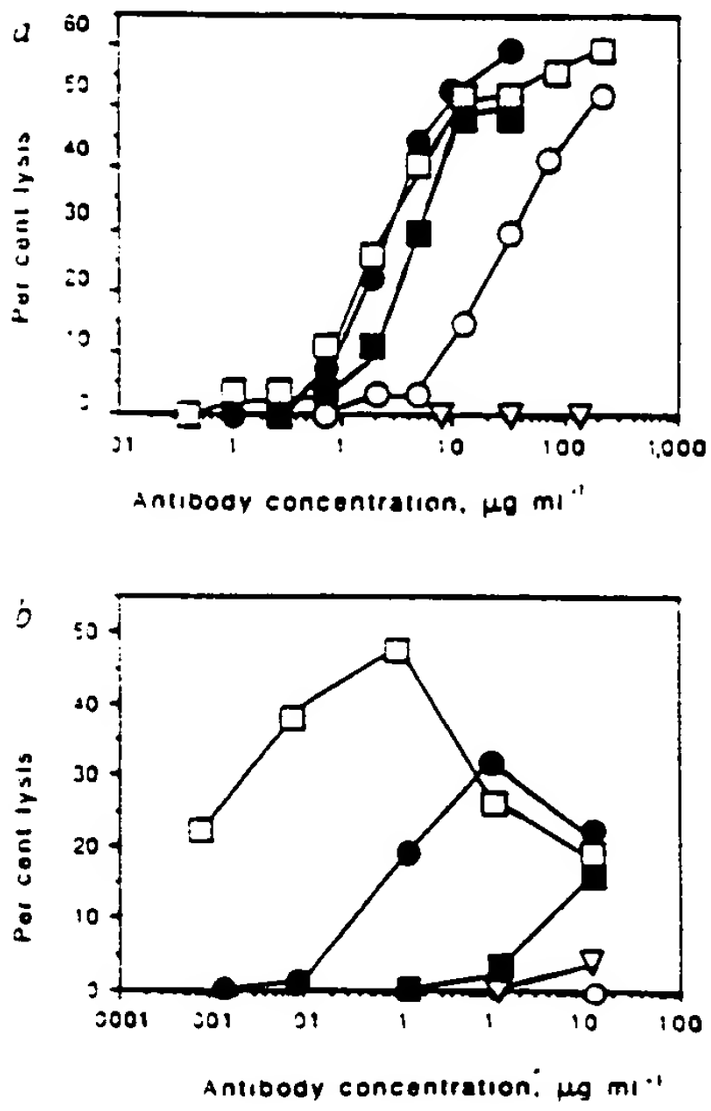

Fig. 4 *a*, Complement lysis and *b*, ADCC for antibodies wi light-chain and rat heavy-chain variable domain attached to hu IgG1 (□), IgG2 (○), IgG3 (■), or IgG4 (▽) isotypes. Lysis the YTH 34.5HL antibody (●) is also shown.
Methods. Antibody was collected from cells in stationary p concentrated by precipitation with ammonium sulphate and d ted into phosphate buffered saline (PBS). Antibodies bound t CAMPATH-1 antigen-coated on microtitre plates, were as in ELISA directed against the rat κ light chain[35], and each adj to the same concentration. The antibodies were assayed in cor ment lysis (Table 1) and ADCC with activated human perip blood mononuclear cells[35,46]. Briefly, 5×10^4 human perip blood cells were labelled with ^{51}Cr and incubated for 30 m room temperature with different concentrations of antibody. E antibody was removed and a 20-fold excess of activated cells a as effectors. After 4 h at 37 °C cell death was estimated by release.

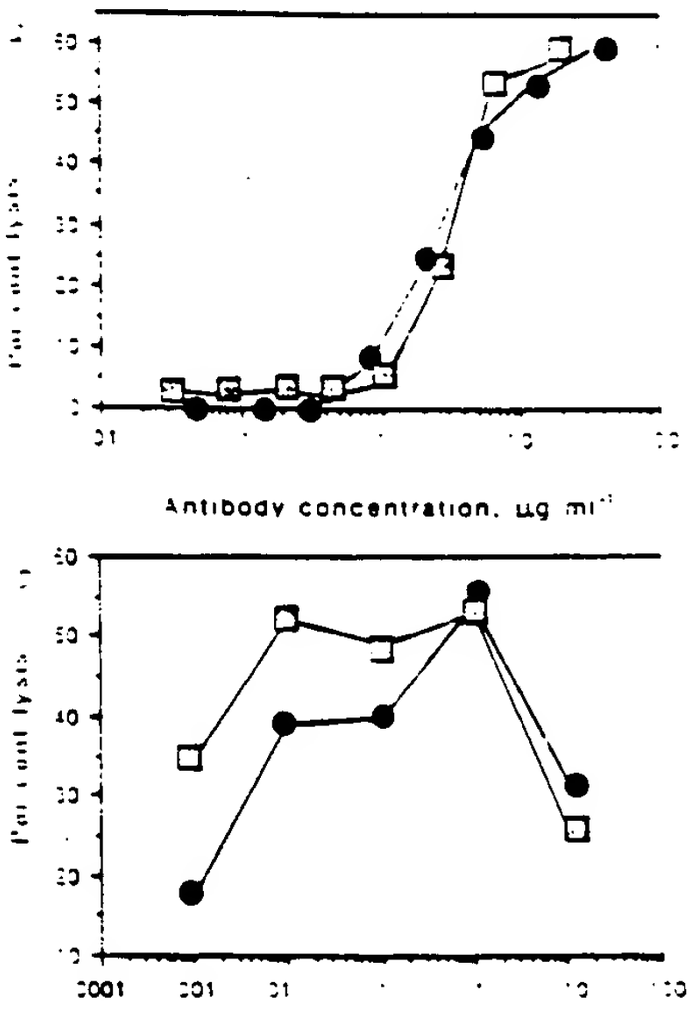

Fig. 5 *a*, Complement lysis and *b*, ADCC of the reshaped human (▩) and rat YTH 34.5HL (●) antibodies. Antibody HuVHCAMP (Ser27 → Phe, Thr30 → Ser)-HuIGG1, HuVLCAMP-HuIGK was purified from supernatants of cells in stationary phase by affinity chromotography on protein-A Sepharose. The yield (about 10 mg l^{-1}) was measured spectrophotometrically. Complement and ADCC assays were performed as in Fig. 4.

Light chain

In stage 3 (Fig. 2), the reshaped heavy chain was completed by attaching the reshaped HuVHCAMP (Ser27 → Phe, Ser30 → Thr) domain to the human IgG1 isotype. The reshaped light-chain domain HuVLCAMP was attached to the human Cκ domain. The two clones were co-transfected into the non-secreting rat Y0 myeloma line. The resultant antibody, bound to CAMPATH-1 antigen (data not shown), and proved almost identical to the YTH 34.5HL-G2b antibody in complement lysis (Fig. 5*a*). In cell-mediated lysis the reshaped human antibody was more effective than the rat antibody (Fig. 5*b*). Similar results were obtained with three different donors of target and effector cells (data not shown). Also, the antibody was as effective as YTH 34.5HL-G2b in killing leukaemic cells from three patients with B-cell lymphocytic leukaemia by complement-mediated lysis with human serum. Thus, by transplanting the hypervariable regions from a rodent to a human antibody of the IgG1 suotype we have reshaped the antibody for therapeutic application.

Prospects

The availability of a reshaped human antibody with specificity for the CAMPATH-1 antigen should permit a full analysis of the *in vivo* potency and immunogenicity of an anti-lymphocyte antibody with wide therapeutic potential. Even if anti-idiotypic responses are eventually observed, considerable therapeutic benefit could be derived from an extended course of treatment. Also, it should be possible to circumvent an anti-globulin response restricted to idiotype by using a series of antibodies with different idiotypes[34]. In principle, the idiotype of the reshaped CAMPATH-1 could be changed by altering the hypervariable regions or the framework regions—evidence from reshaped antibody specific for the hapten nitrophenyl acetate suggests that recognition by anti-idiotypic antisera and anti-idiotypic mAbs is influenced by residues in the framework region[19]. Thus, recycling the hypervariable regions on different human framework regions should change the idiotype, although ultimately it might focus the response directly onto the binding site for the CAMPATH-1 antigen. Although such focusing would be undesirable for CAMPATH-1 antibodies, it could be an advantage for the development of anti-idiotypic vaccines. It is likely that the answers to some of these questions will emerge from the use of this reshaped antibody in therapy.

We thank J. Foote for the synthetic gene encoding the framework regions of a human light chain; P. Leder, T. R. Rabbitts, T. Honjo, M. P. Lefranc respectively for clones encoding the constant regions of human κ-chain, human IgG2 and IgG4, human IgG1, human IgG3; G. Hale for CAMPATH-1 antigen and for advice; M. Bruggemann and M. S. Neuberger for subclones of the heavy-chain constant regions[35] and for advice; M. Frewin for technical assistance and C. Milstein for encouragement. L.R. is supported by a fellowship from the German 'Sonderprogramm Gentechnologie des DAAD'. The work was supported by the Medical Research Council and by Wellcome Biotechnology Ltd. 'CAMPATH' is a trademark of The Wellcome Foundation Ltd.

Received 3 December 1987; accepted 12 February 1988.

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EXHIBIT B-6

Discussion

This study is the largest experience to date with the benzodiazepine antagonist flumazenil in the treatment of HE. The effects of the drug were assessed clinically and by SEP recordings. The late components of cortical SEPs (peaks N1 and P1) appear to be highly sensitive indicators of cortical dysfunction in HE.[1] The results indicate that flumazenil may improve the HE that complicates both acute and chronic liver failure. Flumazenil treatment was associated with improvement in neurological status in 60% of episodes of HE; with one exception improvement occurred within a few minutes to an hour of drug administration. The speed of these responses contrasts with the interval of several hours that is typically necessary before HE improves after conventional therapies. The response to flumazenil in benzodiazepine intoxication is also very rapid.[2]

The 60% improvement rate may even underestimate the potential efficacy of flumazenil in the treatment of HE since most of the patients in this study had been encephalopathic for many days before flumazenil treatment and had not responded to conventional therapy. Furthermore all 5 patients with clinical evidence of increased intracranial pressure due to brain oedema did not respond to flumazenil. 1 of these patients improved after treatment with mannitol. The remaining 4 died within 3 days of flumazenil administration.

In 3 of the 12 episodes responding to flumazenil there was an exacerbation of HE 0·5–4 h after stopping treatment. This transient effect of the drug is consistent with its pharmacokinetics.[3,4] To achieve a sustained response continuous administration of the drug over longer periods may be necessary. Although these 12 episodes improved, no patient regained normal brain function at the end of treatment. The possibility that larger doses or a longer duration of treatment would have achieved complete improvement seems unlikely since, in benzodiazepine intoxication, much lower doses are sufficient for recovery.[4] In addition an increased GABA-ergic tone may be only one of many abnormalities of brain function in patients with liver failure and correction of this particular abnormality may therefore induce incomplete improvement.

The mechanism by which flumazenil improves HE is uncertain. One possibility is displacement of an endogenous benzodiazepine-like substance from the GABA$_A$-benzodiazepine receptor. The presence of such a substance was suggested in the brains of animals with HE and in cerebrospinal fluid of patients dying with HE.[5]

This study was supported by the Fonds zur Förderung der wissenschaftlichen Forschung (P 6169 M). Flumazenil was provided by Hoffmann-La Roche, Basel, Switzerland.

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THE LANCET, DECEMBER 17, 1988

Preliminary Communication

REMISSION INDUCTION IN NON-HODGKIN LYMPHOMA WITH RESHAPED HUMAN MONOCLONAL ANTIBODY CAMPATH-1H

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Summary A genetically reshaped human IgG1 monoclonal antibody CAMPATH-1H was used to treat two patients with non-Hodgkin lymphoma. Doses of 1–20 mg daily were given intravenously for up to 43 days. In both patients lymphoma cells were cleared from the blood and bone marrow and splenomegaly resolved. One patient had lymphadenopathy which also resolved. These effects were achieved without myelosuppression, and normal haemopoiesis was restored during the course of treatment, partially in one patient and completely in the other. No antiglobulin response was detected in either patient. CAMPATH-1H is a potent lympholytic antibody which might have an important use in the treatment of lymphoproliferative disorders and additionally as an immunosuppressive agent.

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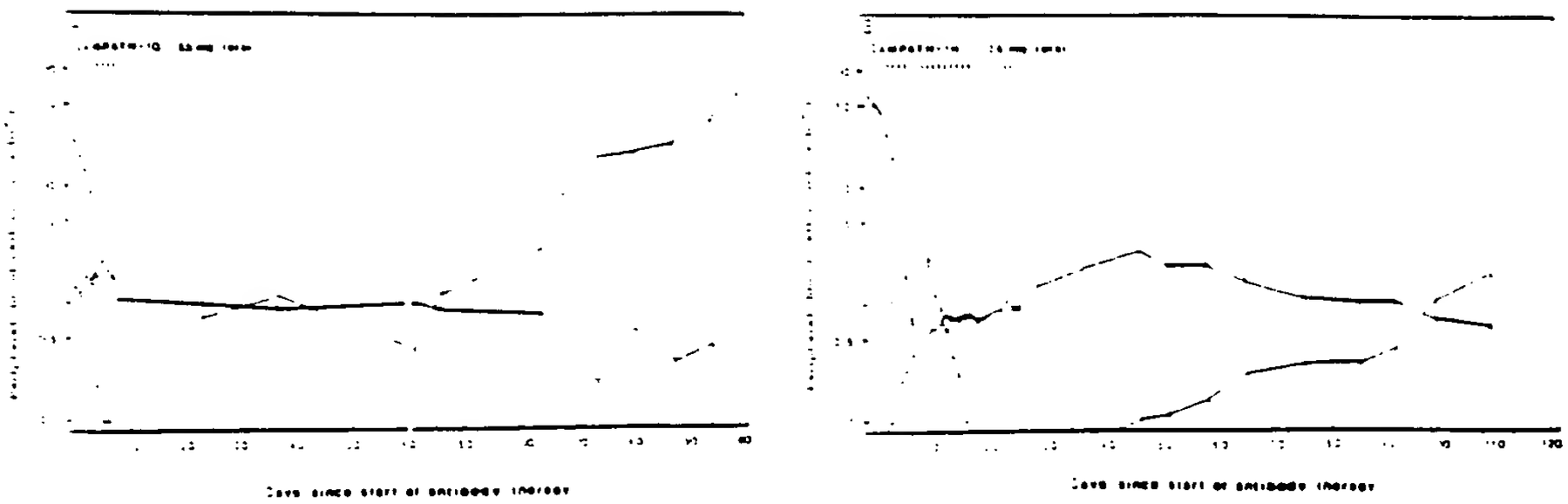

Fig 1—Effect of CAMPATH-1G (A) and CAMPATH-1H (B) on blood counts in patient 1.

▲ = lymphocytes; △ = neutrophils.

INTRODUCTION

TUMOUR treatment by passive serotherapy has had a long and largely unsuccessful history.[1] The advent of monoclonal antibodies gave fresh impetus to this approach, but results with unmodified antibodies are generally unremarkable. Efforts to enhance activity in vivo are now largely focused on the conjugation of antibodies to toxins or radionuclides. However, we are convinced that physiological effector mechanisms are still among the most potent and have tried to find the optimum combinations of antibody specificity and isotype to exploit them fully.

One possible specificity is the CAMPATH-1 antigen.[2] It does not readily undergo modulation and is abundantly expressed on virtually all lymphoid cells and monocytes, but not on other cell types.[2,3] These properties make it a potential target for treatment of lymphoid malignant disorders and for immunosuppression. Several rat IgM and IgG antibodies to this antigen have been produced.[4,5] The IgM (CAMPATH-1M) is intensely lytic with human complement and is widely used for depletion of T cells from bone marrow to prevent graft-versus-host disease.[6,7] The IgG2b (CAMPATH-1G) is the most potent for cell depletion in vivo,[4] probably because it binds to human Fc receptors and can activate the complement system.[3] Patients with lymphoid malignant disorders treated with CAMPATH-1G (25–50 mg/day for 10 days) showed pronounced reduction in lymphoid infiltration of blood and bone marrow and improvement of splenomegaly.[8] However, treatment with rat antibody is likely to be limited by an antiglobulin response. This problem should be reduced or eliminated by use of a human antibody. A reshaped human antibody (CAMPATH-1H) has been constructed—the hypervariable regions of the rat antibody were transplanted into normal human immunoglobulin genes.[9] Human IgG1 was chosen since it had greater activity than other human isotypes both in complement lysis and in cell-mediated killing.[9–11]

Here we describe the use of CAMPATH-1H to treat two patients with non-Hodgkin lymphoma. Although it was possible to continue treatment for up to 6 weeks without the development of a neutralising antiglobulin response, the main point of this report is to describe the efficacy of the antibody in clearing large masses of tumour cells. This is the first report of treatment with a fully reshaped human monoclonal antibody.

PATIENTS AND METHODS

Approval for the use of monoclonal antibodies was given by the ethical committee of Addenbrooke's Hospital and written consent was obtained from both patients.

Antibodies were obtained from culture supernatant of cells growing in a hollow fibre bioreactor (Acusyst-J; Endotronics). CAMPATH-1G was purified by precipitation with ammonium sulphate; CAMPATH-1H was purified by affinity chromatography on protein-A–Sepharose. They were dissolved in phosphate-buffered saline, sterile filtered, and tested for pyrogen and sterility. Patients were premedicated overnight and antibody, diluted in 500 ml saline, was infused over 2–4 h.

CAMPATH-1 expression on tumour cells was measured by flow cytometry and complement-mediated lysis.[2,3,8] Serum concentrations of CAMPATH-1H were measured by immunofluorescence with normal lymphocytes.[8] Southern blot analysis with an immunoglobulin J_H probe was used to detect residual tumour cells in DNA extracted from mononuclear fractions of bone marrow.[8] Antiglobulin responses were sought by two techniques. The first was a solid-phase enzyme-linked assay using microtitre plates coated with CAMPATH-1H. After incubation with patients' serum samples, the assay was developed with biotin-labelled CAMPATH-1H followed by streptavidin-peroxidase. A mixture of monoclonal mouse antibodies against human IgG was used as a positive control and 500 ng/ml of this mixture could be detected. In the second assay, patients' serum samples were mixed with red cells coupled with CAMPATH-1H.[12] Agglutination by 5 ng/ml of the control mixture could be detected. Immunoglobulin allotypes were determined by means of standard reagents and techniques from the Central Laboratory of the Netherlands Red Cross blood transfusion service.

RESULTS

Patient 1

A 69-year-old woman presented in 1983 with acute appendicitis. Massive splenomegaly was found (table) and the bone marrow was heavily infiltrated with lymphocytes, some of which had cleft nuclei and a single nucleolus. There was weak membrane expression of IgM-kappa. Computed tomography scan showed splenomegaly but no lymphadenopathy. Grade I, stage IVA non-Hodgkin lymphoma in leukaemic phase was diagnosed. Between 1983 and 1987 the patient received oral and intravenous chemotherapy with combinations of cyclophosphamide, vincristine, prednisolone, and chlorambucil, which induced partial responses, the minimum level of marrow infiltration being 40%. Two courses of splenic radiotherapy were given.

but the second in March 1988 was curtailed since the spleen grew larger during the course.

In September 1987 the disease progressed with increases in blood lymphocytes (2·1 × 10⁹/l) and spleen size. The patient was treated with CAMPATH-1G for 5 days (fig 1A). This treatment completely cleared lymphoma cells from blood and marrow but only partially reduced spleen size. CAMPATH-1G induced fever, nausea, and vomiting, and the treatment was stopped on day 5 when it resulted in severe bronchospasm. Such severe reactions have not been seen in twenty-one other patients who have received similar doses.) Reappearance of lymphoma cells in the blood was initially slow and the spleen size did not change for 5 months but there was little recovery of normal haemopoiesis. In March 1988 the patient began to lose weight and experienced drenching night sweats. The spleen enlarged and lymphoma cells reaccumulated in the blood. They had similar phenotype and identical rearranged immunoglobulin J_H fragments to those seen before treatment. Marrow aspirate and trephine showed complete replacement of normal marrow by lymphoma cells (fig 2A); the patient became dependent on red-cell transfusions and was absolutely neutropenic.

The patient's serum did not block binding of CAMPATH-1H or CAMPATH-1G to normal lymphocytes and the tumour cells were still sensitive to these antibodies in vitro, so we decided to treat her with CAMPATH-1H. The starting dose was 1 mg daily and, since it was well tolerated, the dose was increased to a maximum of 20 mg/day, though the usual dose was 4 mg/day owing to the small amount available. In all the patient received 126 mg over 30 days. The response was prompt; in 6 days the night sweats had abated, by day 10 there was pronounced reduction in splenomegaly and recovery of blood neutrophils, and by day 18 lymphoma cells were cleared from the blood (fig 1B). On day 28 a bone marrow aspirate and trephine were hypocellular but showed active myelopoiesis and erythropoiesis and no lymphoid cells (fig 2B). No CAMPATH-1-positive cells could be detected by flow cytometry. DNA from the mononuclear marrow cells was germline when probed with an immunoglobulin J_H probe under conditions where clonal rearrangements could be detected in 0·2% of cells. Thus, we conclude that lymphoma cells were cleared from the marrow. The spleen volume was reduced about eight-fold (fig 3A, B), although it was still slightly larger than normal.

Other than fever occurring about 1 h after the end of antibody infusions there were no adverse effects of antibody treatment until the 5th week, when severe rigors occurred after each infusion. No antiglobulin response could be detected and the rate of clearance of antibody from the serum was unchanged. For the next 3 weeks the patient continued to experience occasional fever and rigors. She was given oral cotrimoxazole because of her lymphopenia, but no infective cause of these symptoms could be found.

In the next 4 months lymphocytes, which appeared morphologically normal, slowly reappeared in the blood (up to 0·2 × 10⁹/l). They did not show the characteristic rearranged immunoglobulin fragments, and both CD3-positive and CD19-positive cells were present (table). Serum immunoglobulin levels, which had been very low since presentation, have risen towards normal (table). A marrow aspirate and trephine taken 50 days after the end of treatment were again hypocellular but had no lymphomatous infiltration. This marrow sample contained

PATIENT CHARACTERISTICS BEFORE AND AFTER TREATMENT WITH CAMPATH-1H

	Patient 1		Patient 2	
	Before	After*	Before	After*
Lymphadenopathy†	None	None	1500 Retrocrural, para-aortic	None
Bone marrow				
Lymphoma cells	+3	−	+2	−
Southern blot analysis J_H fragment	R R	G G	R R	G G
Peripheral blood				
Haemoglobin (g/dl)	9·7	9·9	11·2	12·0
Reticulocytes (× 10⁹/l)	0·1	0·5	ND	ND
Platelets (× 10⁹/l)	37	52	110	153
Lymphocytes (× 10⁹/l)	2·1	0·1	0·7	1·1
Neutrophils (× 10⁹/l)	1·1	2·0	4·0	4·3
Monocytes (× 10⁹/l)	0·1	0·04	0·5	0·5
Blood lymphocyte phenotype (%)				
CD19	97	2·3	93	4·5
CD3	1·1	2·2	5	9·0
CAMPATH-1M	96	ND	95	ND
CAMPATH-1H	94	ND	97	ND
Serum immunoglobulins				
IgM	<0·3	0·5	<0·3	0·7
IgA	<0·5	<0·5	<0·5	0·5
IgG	5·5	8·2	3·2	4·7
Bence-Jones	None	None	− −	None

*Made shortly after end of antibody treatment, except for lymphocyte phenotyping and serum immunoglobulins, which were assessed 6 weeks later.
†By computed tomography.
ND = not done.

4% CAMPATH-1-positive cells and showed some oligoclonal rearrangements of immunoglobulin genes. However, by day 100, lymphoma cells were again detected in the blood and the spleen size had started to increase. A second course of 12 days' therapy with CAMPATH-1H was completed with similar therapeutic benefit to the first and no adverse effects. Since the main reservoir of disease in this patient appeared to be the spleen, splenectomy was carried out at the end of this second course of treatment. At that time no tumour cells could be detected in blood or marrow. The patient is now well 37 days after the splenectomy. The lymphocyte count is low but she has normal neutrophil, platelet, and red-cell counts.

Patient 2

A 67-year-old man presented in April 1988 with splenic pain; there was 12 cm splenomegaly, and computed tomography scan of thorax and abdomen revealed retrocrural and para-aortic lymphadenopathy, the largest node measuring 3 cm in diameter (fig 3C). A blood count revealed 36·6 × 10⁹ lymphocytes/ml, the majority being lymphoplasmacytoid cells which expressed surface IgG-kappa and were characterised by large cytoplasmic periodic-acid-Schiff-positive vacuoles which could be intensely stained by anti-IgG. A marrow aspirate contained 72% lymphomatous cells (fig 2C). DNA from blood mononuclear cells showed biallelic rearrangement of immunoglobulin J_H genes but was germline with various T-cell receptor and oncogene probes. The lymphoma cells expressed the CAMPATH-1 antigen in amounts comparable with normal lymphocytes but were more resistant to complement-mediated lysis. Stage IVA grade I

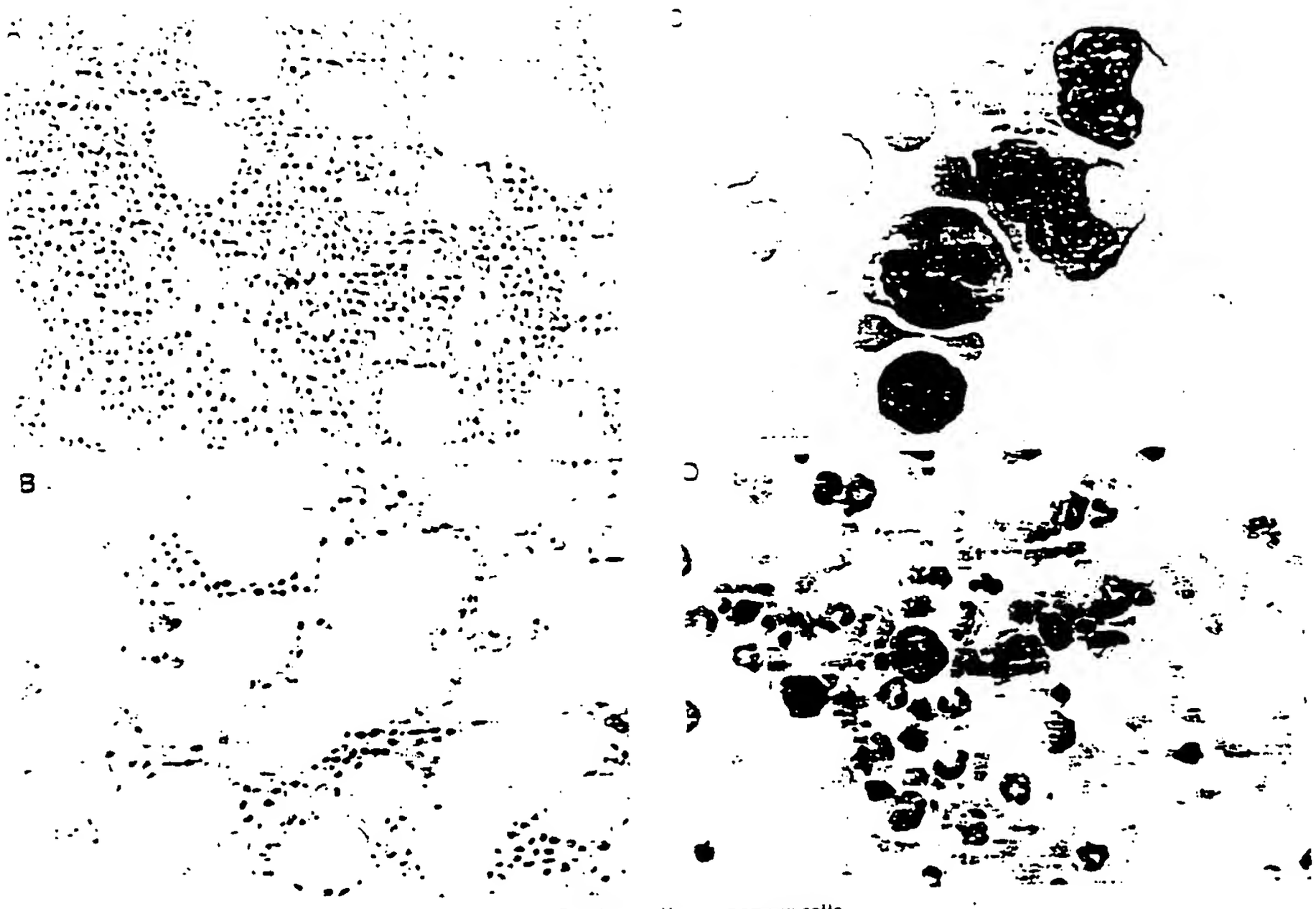

Fig 2—Cytology of bone marrow cells.

A = patient 1 trephine before treatment with CAMPATH-1H; B = patient 1 trephine on day 43 (ie, 16 days after treatment); C = patient 2 aspirate before treatment with CAMPATH-1H; D = patient 2 aspirate on day 78 (ie, 35 days after treatment). Reduced by 58% from × 100 (A,B), × 1000 (C), × 400 (D).

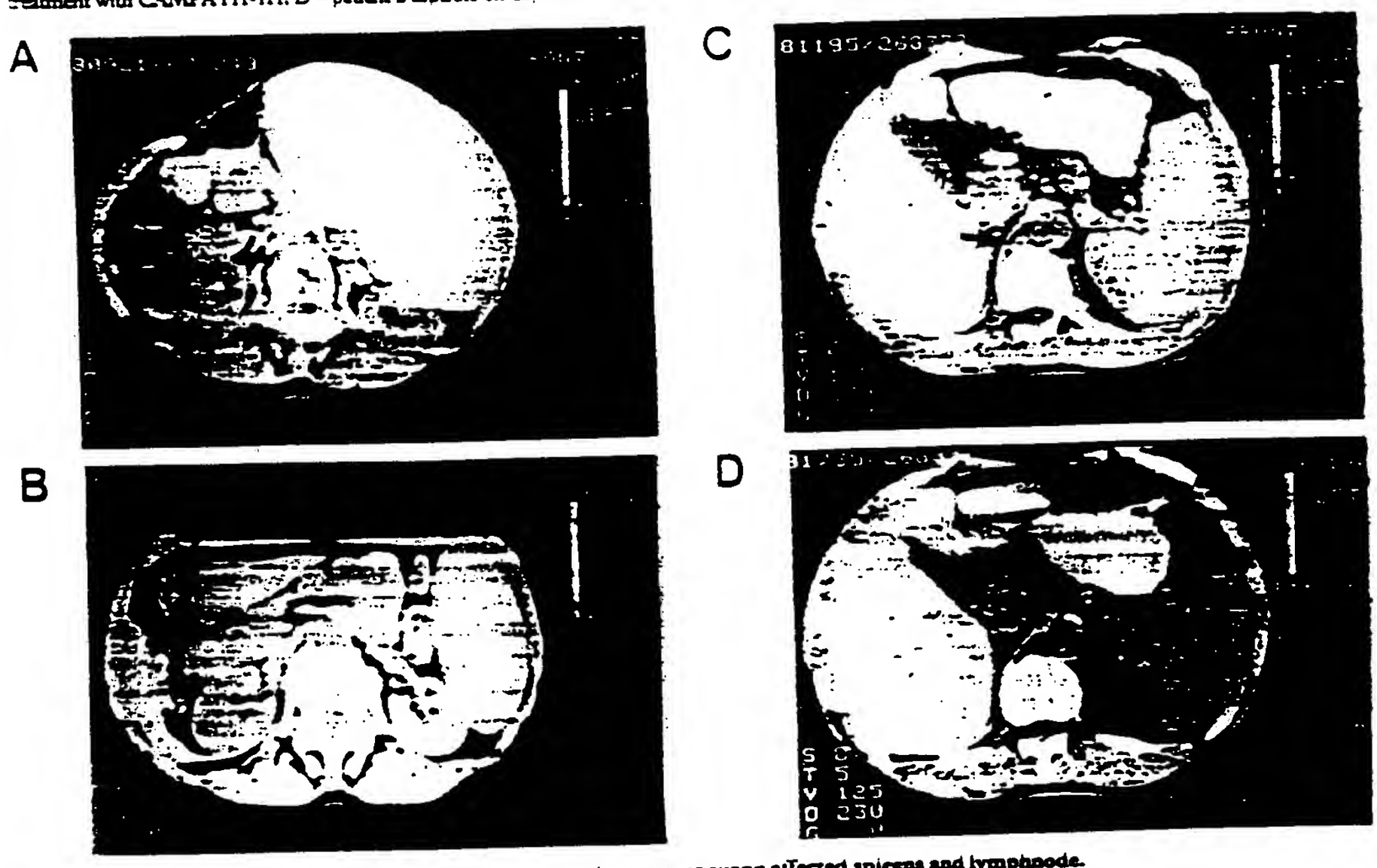

Fig 3—Computed tomography scans showing affected spleens and lymphnode.

A = patient 1 before treatment with CAMPATH-1H; B = patient 1 on day 57; C = patient 2 before treatment with CAMPATH-1H (retrocrural node arrowed); D = patient 2 on day 51.

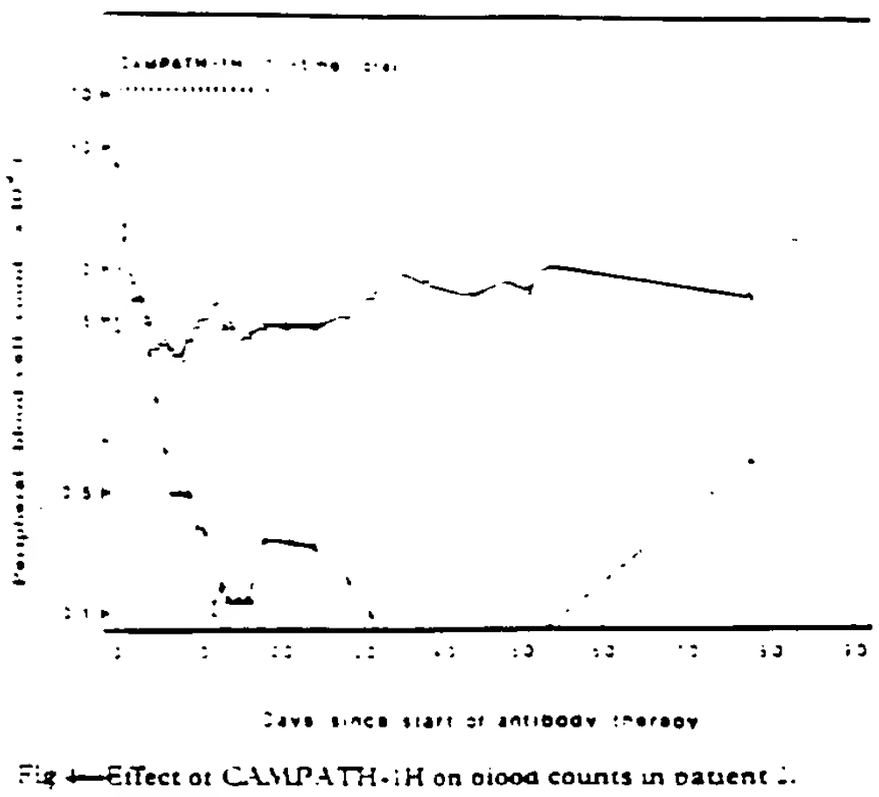

Fig 4—Effect of CAMPATH-1H on blood counts in patient 2.

▲ = lymphocytes; ● = leucocytes.

lymphoplasmacytoid non-Hodgkin lymphoma was diagnosed.

The patient was offered CAMPATH-1H as primary therapy and received a total of 85 mg over 43 days. This resulted in clearance of the lymphoma cells and normal lymphocytes from blood (fig 4) and marrow (fig 3D), resolution of splenomegaly, and improvement in the lymphadenopathy. A computed tomography scan taken 8 days after the end of antibody treatment was normal (fig 3D). A bone marrow aspirate taken at the same time showed active haemopoiesis but no lymphoma cells, and no CAMPATH-1-positive cells could be detected by flow cytometry. DNA from the mononuclear fraction of this marrow showed only germline configuration when probed with the immunoglobulin J_H probe. By day 78 morphologically normal blood lymphocytes began to reappear and none of the vacuolated cells could be seen. The patient remains well and off therapy.

Some toxic effects of CAMPATH-1H were observed. The first dose was stopped after 3 mg had been given because of hypotension, possibly caused by tumour lysis. This problem was subsequently avoided by giving smaller doses at a slower rate and when lymphoma cells had been cleared from the blood. the dose was increased to a maximum of 8 mg over 4 h without any effect on blood pressure. Nevertheless, all doses induced fever (up to 38·5°C), and malaise for up to 36 h, but these were not severe enough to stop antibody treatment which, after the first week, was given on an outpatient basis. Treatment was stopped after 43 days because of the development of an urticarial rash after two successive antibody infusions.

Half-life of CAMPATH-1H

The concentration of CAMPATH-1H was measured in serum samples taken before and after antibody infusions and at other times throughout treatment. In theory, a dose of 4–6 mg corresponds to about 1 μg/ml in the plasma. In fact we could not detect free antibody till day 4–6, presumably because of rapid uptake by the tumour mass. After that, the rate of clearance was roughly constant, with the concentration being about 30–70% of the theoretical level immediately after infusion and about 5–10% after 24 h. The rate of clearance of CAMPATH-1H was possibly slightly slower than that of the rat CAMPATH-1G,[7] but still much faster than that of normal human IgG1.[8]

Lack of Antiglobulin Response

The isotype of CAMPATH-1H is G1m1,2,17,Km3. Patient 1 was G1m1,2,17,Km3, and patient 2 was G1m3, Km3, so both could theoretically have made an anti-isotype response as well as a response to the hypervariable regions. However, we failed to detect any antiglobulin to CAMPATH-1H either by the solid-phase enzyme-linked assay or by the more sensitive haemagglutination assay. In addition, the rate of clearance of CAMPATH-1H did not change and free antibody could be detected for up to 8 days after the last dose had been given. It is possible that the reactions experienced at the end of the course of treatment could have been provoked by contaminating non-human proteins in the antibody preparation.

DISCUSSION

The remissions achieved in these two patients show that it is possible to clear large numbers of tumour cells with small amounts of an unmodified monoclonal antibody. The effects in the first patient were far superior to the results of previous chemotherapy and radiotherapy. The selective lysis of lymphoma cells with recovery of normal haemopoiesis during the course of treatment was an important advantage, which allowed treatment to be given largely on an outpatient basis. We believe the choice of antibody specificity and isotype is important: indeed, it may be why we had more success than previous efforts with other monoclonal antibodies.[1–6] The CAMPATH-1 antigen seems to be a good target because it is widely distributed and abundant, and does not suffer from antigenic modulation.[2,3,8,17] This study shows that, as predicted, human IgG1 can bring about cell lysis in vivo, though we cannot yet assess the relative importance of humoral or cellular mechanisms. There was no change in serum complement levels (CH50, C3, or C4 components) during antibody treatment (data not shown), but this does not exclude a role for C3 in cell clearance.

Although the two patients did not make any serologically detectable antiglobulin response, it would be premature to draw general conclusions about the immunogenicity of human monoclonal antibodies, since CAMPATH-1H itself is probably immunosuppressive and the patients were already immunosuppressed as a result of their disease. Nevertheless, it was encouraging that two courses of antibody treatment could be given, even in the patient who had previously had unusually severe reactions to the original rat antibody.

The long-term benefit of treatment with CAMPATH-1H can only be assessed in a much larger trial when it would probably be combined with more conventional chemotherapy and radiotherapy. It may have wider applications as an immunosuppressive agent for transplantation and possibly autoimmune disease, since we already know that the rat antibody CAMPATH-1G is a potent immunosuppressant in the short-term.

We thank the patients and families, nursing staff, medical colleagues, and Prof F. G. J. Hayhoe for their cooperation, encouragement and support; and Dr D. Gilmore, Dr H. S. Kruger-Gray, Prof R. R. A. Coombs, Mark

...ers and Caroline Markland for their help. This work was supported by the Medical Research Council, Wellcome Biotech Ltd, and St Thomas's College Studentship to M. J. S. D. CAMPATH-1 is a trade mark of Wellcome Foundation.

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Reviews of Books

Worse than the Disease: Pitfalls of Medical Progress

Diana B. Dutton with contributions by Thomas A. Preston and Nancy E. Pfund. Cambridge: Cambridge University Press. 1988. Pp 528. £35. ISBN 0-521340233.

Dr Dutton is a sociologist with a special interest in the development of health policy. She clearly shares Lord Salisbury's view that doctors are a variety of expert who require to have their strong wine diluted by very large admixture of insipid commonsense. On the evidence of this book she has a strong case. Four detailed histories of major medical developments are presented. Two of these initiatives caused considerable harm and suffering to a small number of people at enormous cost and without clinical benefit. The American swine flu mass immunisation programme was designed to protect against an epidemic that did not occur and resulted in severe neurological disease in some unlucky recipients. The artificial heart programme consumed vast federal funds over many years and, when tested (probably prematurely) in man, failed to extend life significantly but afforded a few individuals a miserable death. A third development, diethylstilboestrol, was hailed as a wonder drug and widely put to unproven use until serious adverse sequelae were noted in the children of women who had received it. In the fourth case history, the development of recombinant DNA methods, there is no discernible evidence of physical harm, although the safeguards introduced in the early days, after public debate, were brushed aside under commercial and scientific pressures. In the absence of any harmful outcome, this last case is very much the odd man out: I suspect that it is included because of the early public consultation, although this consultation had little effect upon the course of events.

In the first three examples there is an element of being wise after the event. At least some of those involved acted from the purest of motives when there was considerable uncertainty about the paths to be taken. Later, market forces distorted clinical and scientific judgment, precipitating unjustified clinical use together with destruction of necessary action by the regulatory authorities. It is a sorry tale, and if there is one obvious lesson it is that the marketplace is no testing ground for medical innovation of the sort discussed here. Where financial returns are involved, they only too easily corrupt scientific, clinical, and ethical judgment—in ways that are not always obvious to the participants at the time.

Dutton continues to swim against the tide by suggesting that governments must take responsibility for safeguarding society from the consequences of regarding medical developments as saleable commodities. This philosophy she sees as a variant of Tudor Hart's inverse-care law whereby the areas of greatest need attract the least resources. The difficulty here is obvious from one of her case histories—the mass immunisation programme against swine influenza. Here an early warning system was triggered too easily, a President in an election year needed to present a decisive image, and experts lost the courage of their convictions in the face of the high cost of possibly being proved wrong. The result was a programme that would have failed to stem an epidemic even if the epidemic had occurred. Where powerful governmental machinery existed it over-reacted in an incompetent way.

Dutton recognises the shortcomings of governmental machinery. Her solution is public accountability through other mechanisms at local and national level. She recognises the obvious difficulty presented by the way the popular voice is heard at present. This heavenly chorus "sings with a strong upper class accent. Probably about 90% of the people cannot get into the pressure system". One model she sees in a favourable light is the citizens' panel set up by the Cambridge (Massachusetts) City Manager to examine the potential risks of recombinant DNA research at two of the world's leading universities. This unique approach employed non-experts as a jury. At a national level she proposes greater congressional oversight of medical innovation and perhaps the construction of an overall policy-making body within the United States Department

TABLE 1

TABLE 3 SUMMARY OF IMMUNOLOGICAL TESTS ON CAMPATH-1H PREPARATIONS

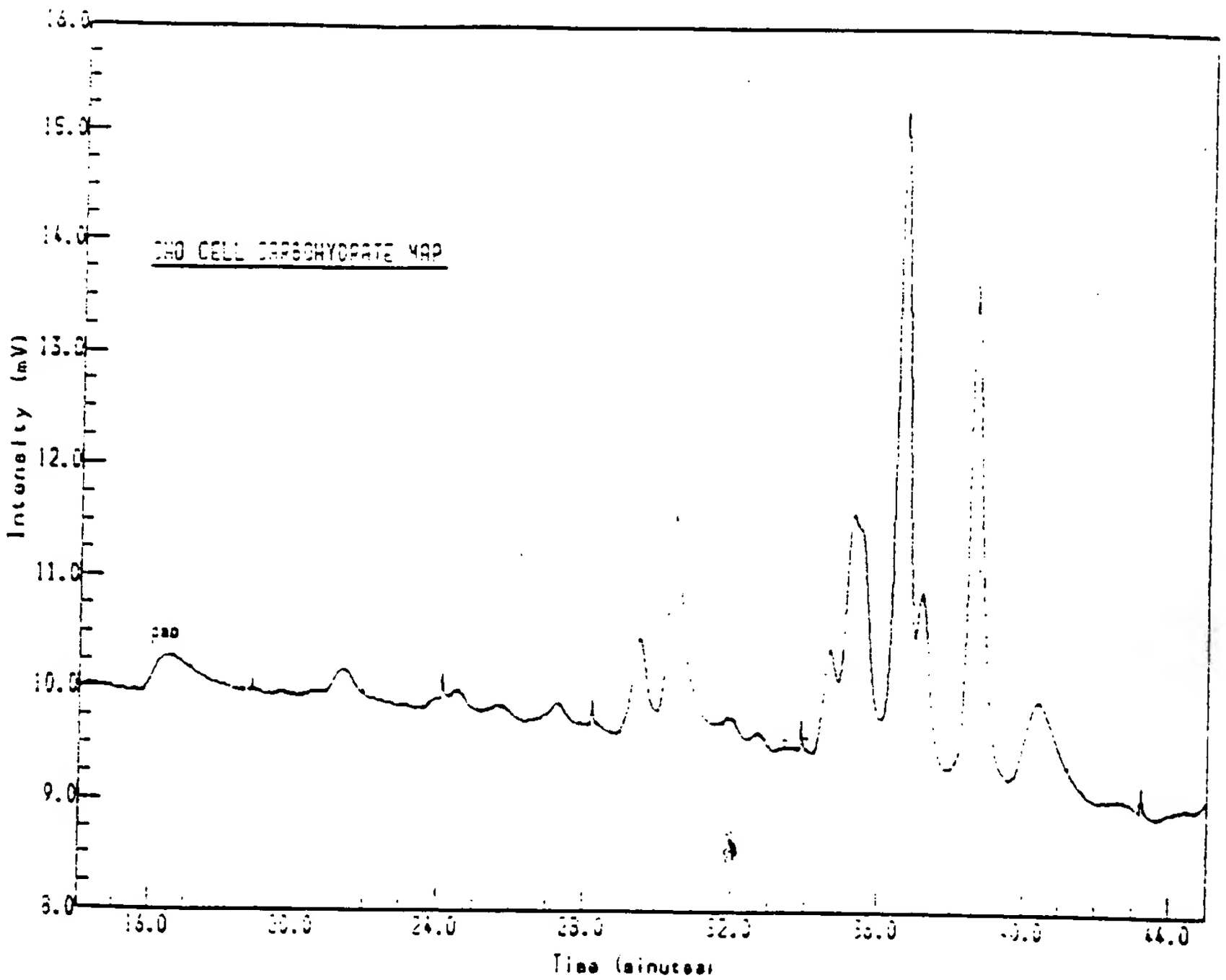
CAMPATH-1H preparation	ELISA (mg/ml)	T-cell[a] binding (U/ml)	Antigen[b] binding (U/ml)	Complement mediated lysis (U/ml)
CHO	1.72	1.82	1.77	1.69
Myeloma	1.83	1.46	1.60	1.42

a T-cell(HUT-78) binding radioassay. Antibody activity (U/ml) is relative to a purified CHO-derived CAMPATH-1H (1mg/ml) standard, defined as having 1000U/ml.

b Soluble CDw52 antigen binding ELISA.

Figure 1

REVERSE-PHASE HPLC [illegible] RELEASED SUGARS



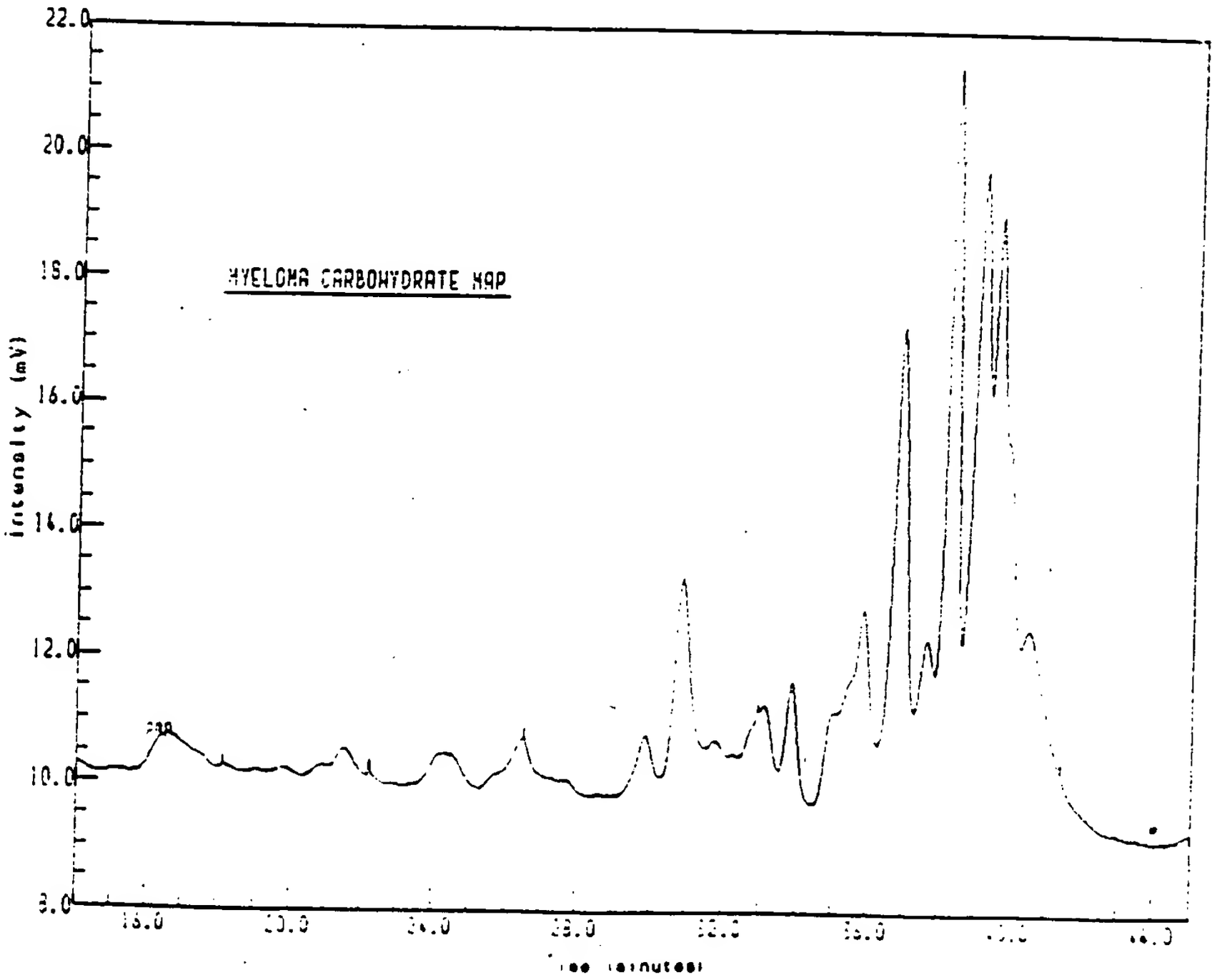
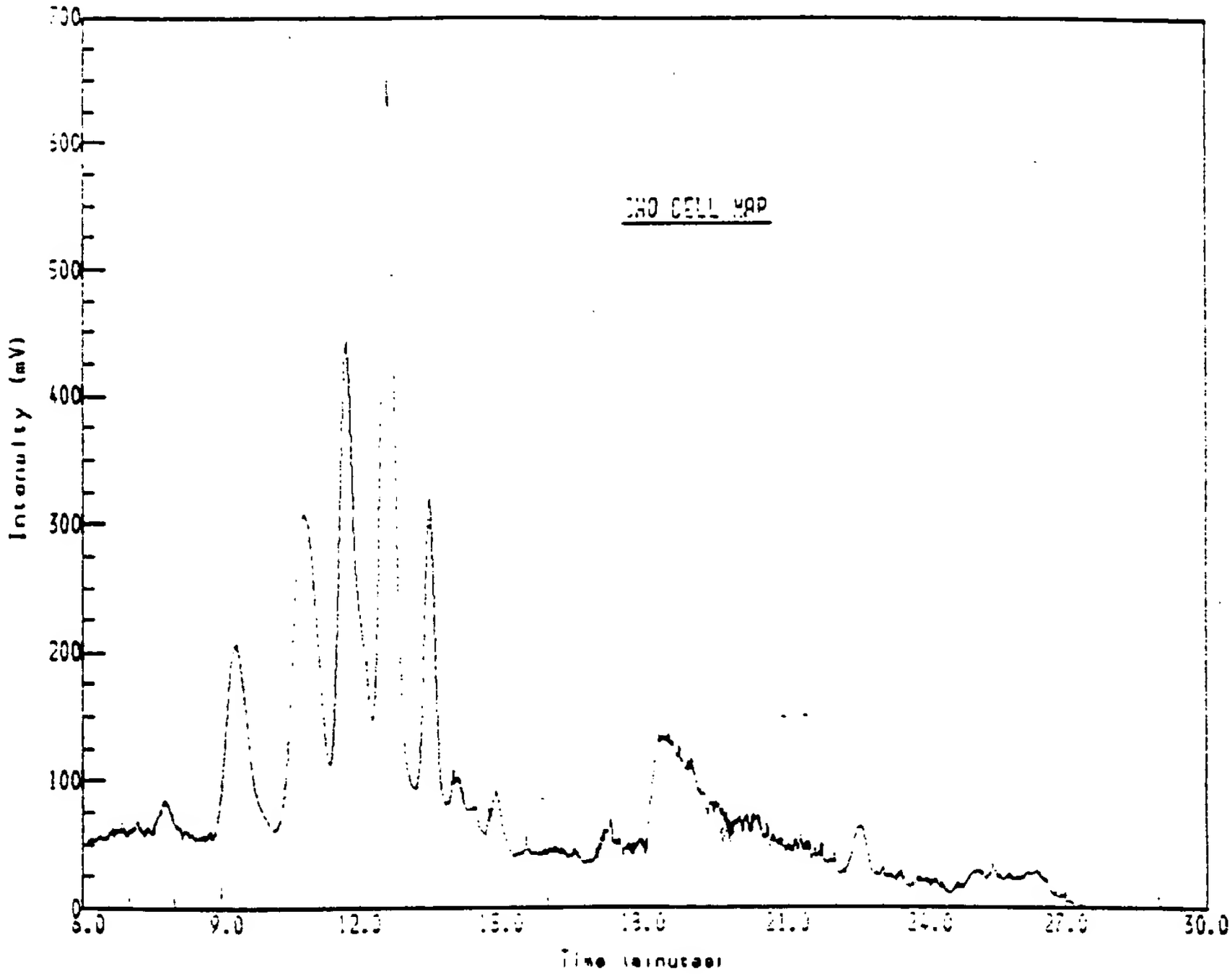


Fig. 1 HPAEC OF THE RELEASED SUGARS



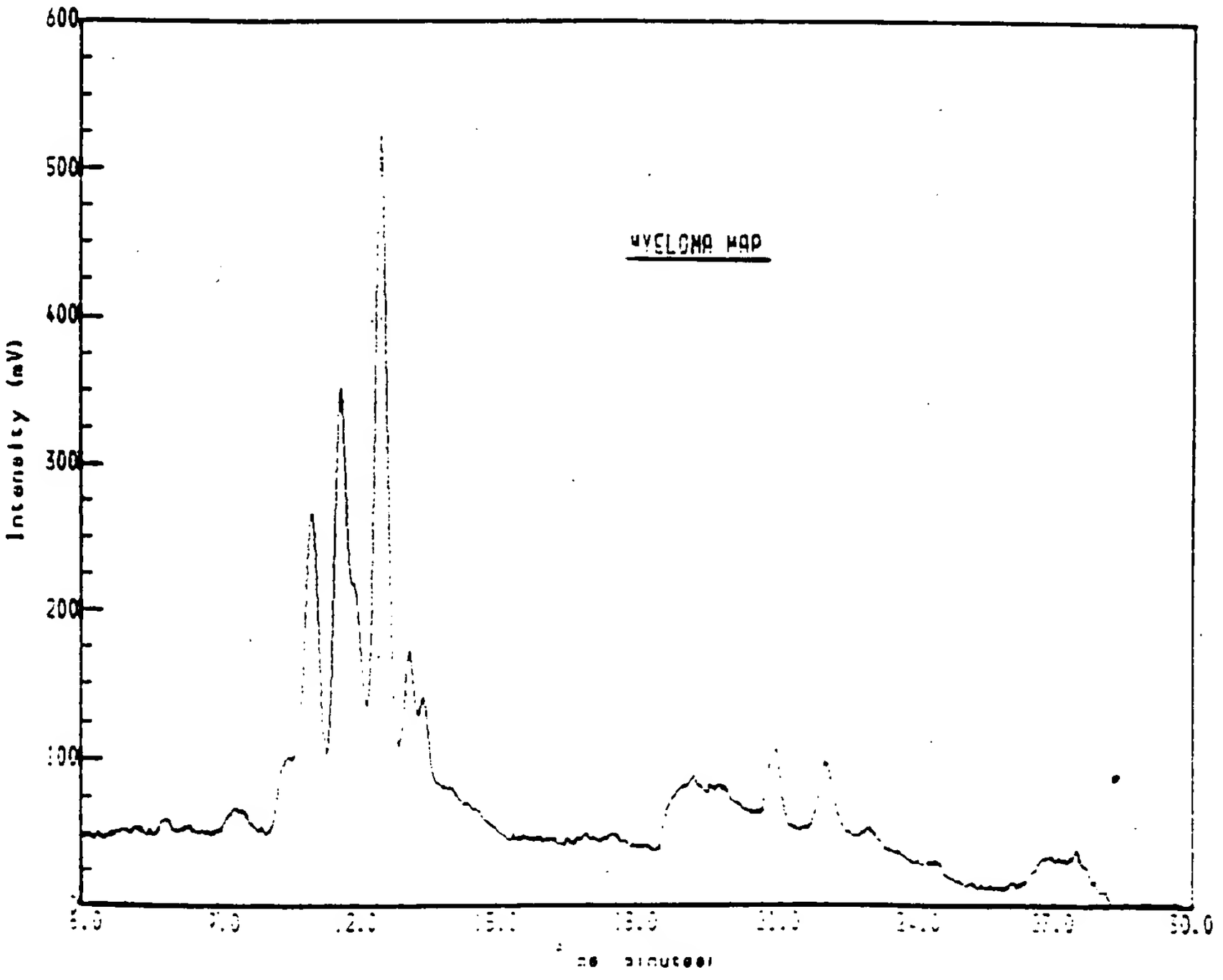


Fig. 3 Assay parallelism between CHO and myeloma CAMPATH-1H

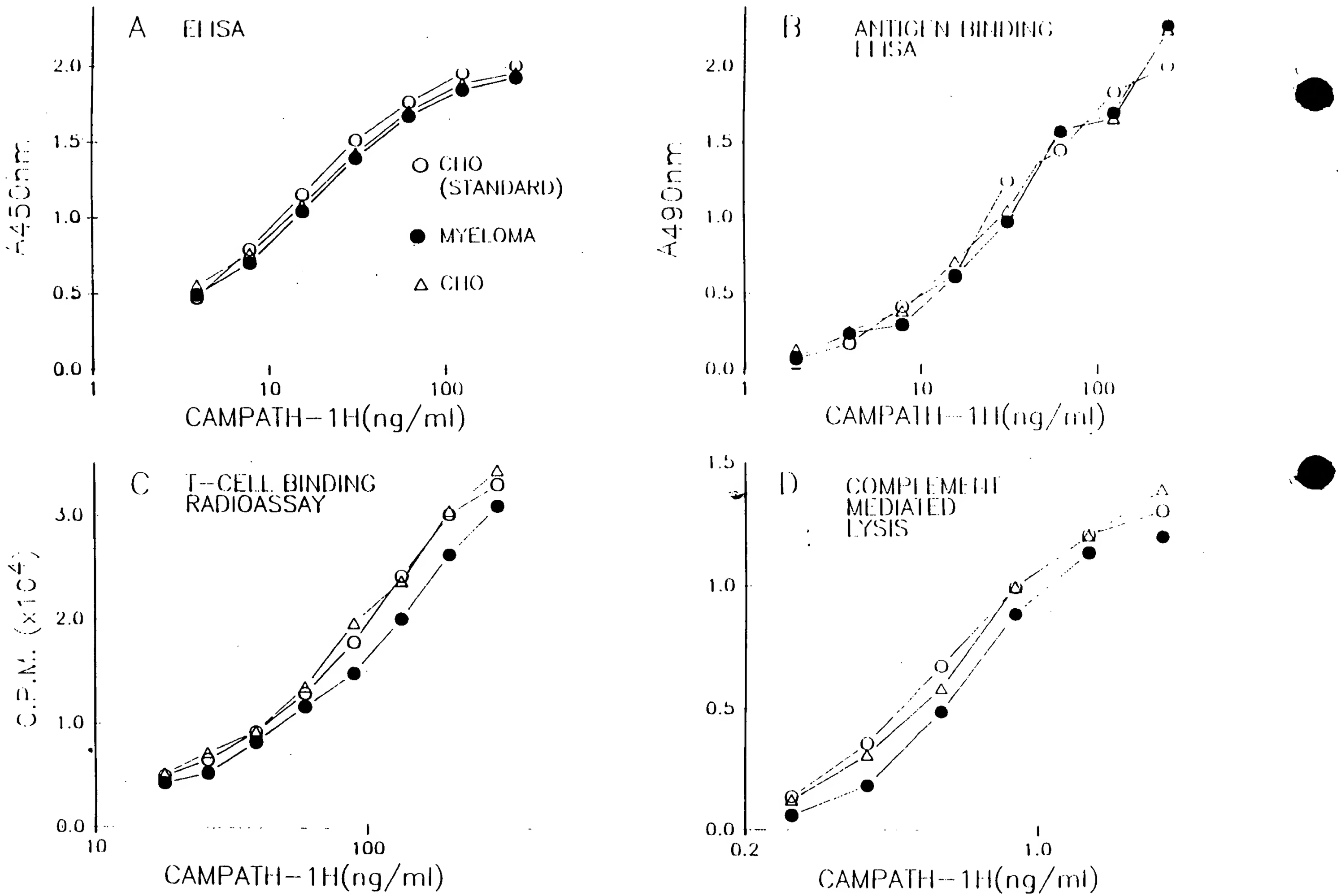
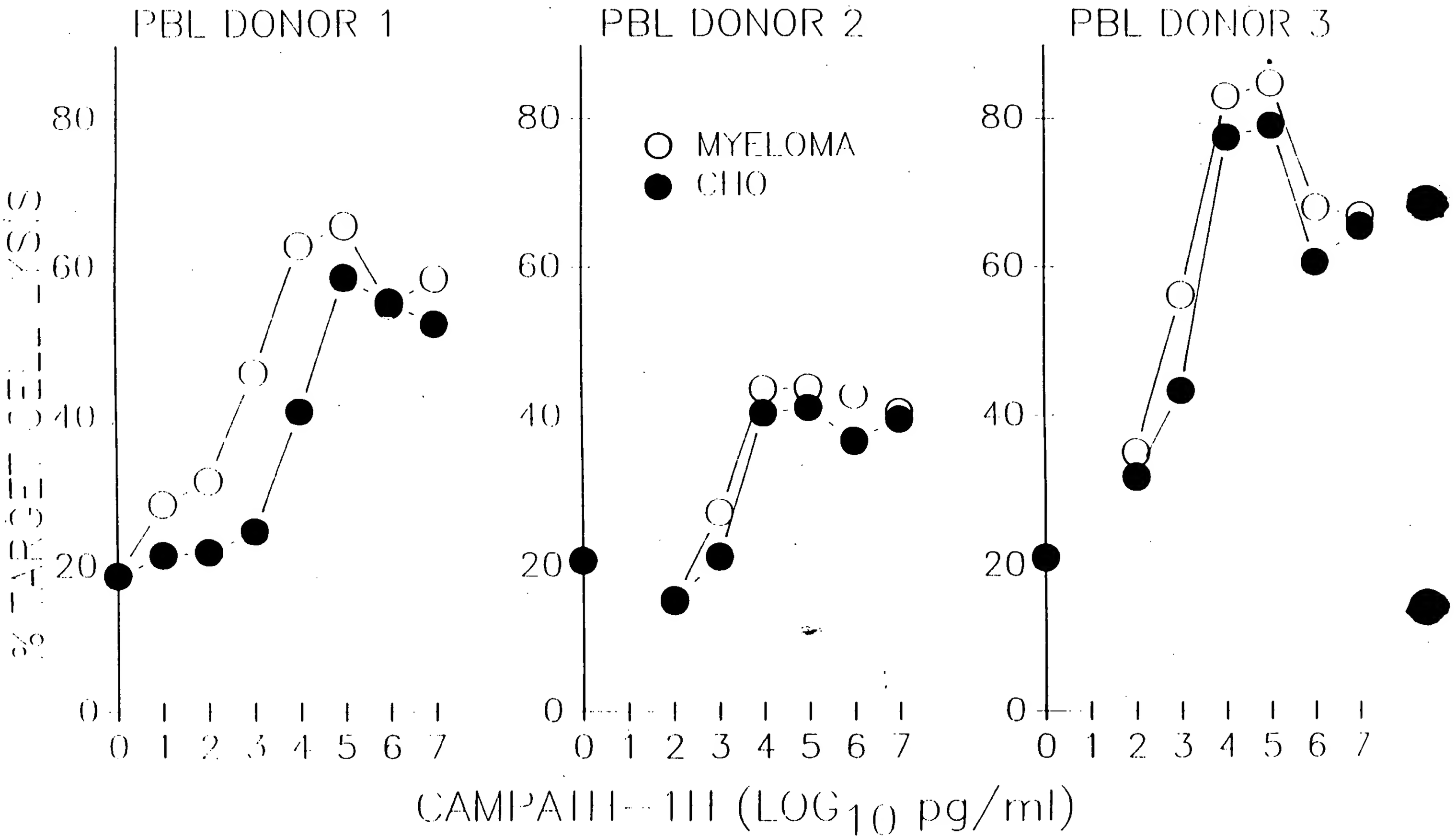


Fig. 4 ADCC assay of CHO and myeloma CAMPATH-1H



Alzheimer's disease: prospects for treatment

SIR,—β-amyloid precursor protein (βAPP) is expressed in increased amounts in neurons that are undergoing synaptogenesis during development, reinnervation, and resprouting[1]—an observation that Dr Murphy (Dec 19/26 p 1512) neglects in his review of the therapeutic prospects for Alzheimer's disease (AD). Investigations of Down's syndrome and head injury[2] suggest that the level of βAPP can rise above the limits of neuronal metabolic capacity and lead to an extracellular deposition of the β-amyloid protein, an event widely believed to precipitate eventual tangle formation.

Ageing is the most important risk factor for AD, and is associated with neuronal resprouting, which leads to an increase in the expression of βAPP. The development of AD-related pathological changes may be regarded as a result of the loss of control of protein expression needed for repair and regeneration of neurons.[3] Neuronal damage, repair, and regeneration are associated with a cascade of proteins, which is termed the acute-phase response and which stimulates protein expression in the brain. The expression of interleukin-1 (IL-1), which is a major component of this response, is raised in both AD and Down's syndrome.[4] IL-1 directly promotes the expression of βAPP.[5] The actions of IL-1 can be specifically blocked by an endogenous protein—the interleukin-1-receptor antagonist (IL1-RA). The importance of the IL-1 system has been recognised in the modulation of systemic disease processes. For example, in Hodgkin's disease[6] and Lyme disease[7] the balance between the levels of IL-1 and IL1-RA have proved of clinical significance.

Preliminary evidence suggests that IL1-RA reduces the IL-1 induced βAPP secretion from cell cultures.[5] This effect has also been demonstrated in vivo in animal models of brain injury.[8] Thus, manipulation and blockade of IL-1 stimulated responses can ameliorate excess production or secretion of βAPP—the critical feature that initiates the pathological cascade in Alzheimer's disease. IL1-RA may have considerable value in the treatment of neurodegenerative disorders such as AD.

Financial support was given by the Haberdashers' Company.

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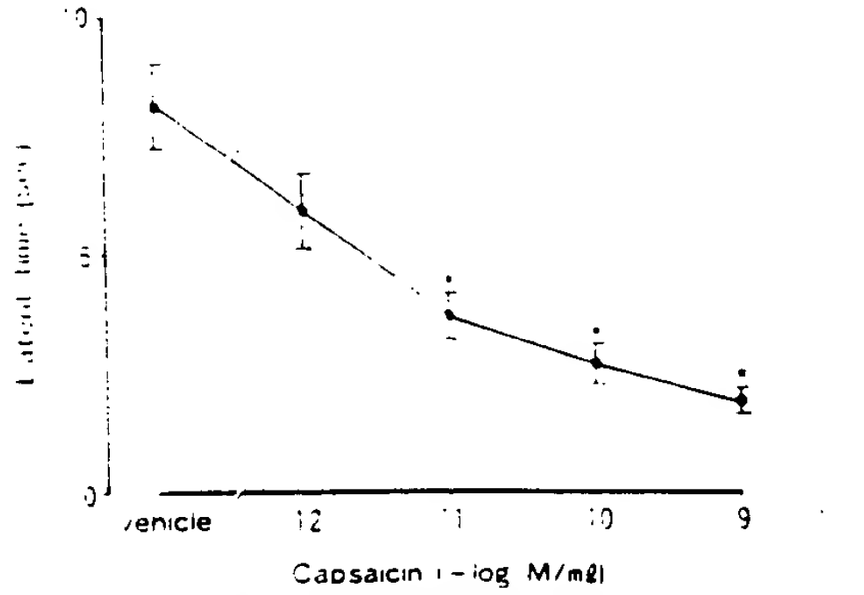
Capsaicin and swallowing reflex

SIR,—Swallowing disorders are common in the elderly and cause significant morbidity and mortality due to aspiration pneumonia. Delayed triggering of the swallowing reflex occurs in patients with swallowing disorders. We have examined the effects of capsaicin, the pungent substance in red peppers that can stimulate sensory nerves,[1] on swallowing reflex in the elderly.

The 20 patients, mean age 76 (SE 2) years, had cerebral thrombosis or dementia due to cerebral arterial sclerosis. Computed tomography revealed various degree of cerebral atrophy and lacunar infarction in all patients. We also studied age-matched controls, mean age 74 (1), who were volunteers and led an active daily life.



Dose-response effects of capsaicin on latent time of swallowing.

*Significant differences from values for vehicle ($p < 0.01$).

To eliminate any diurnal variation in swallowing reflex the challenges were done at the same time of day. The swallowing reflex was induced by a bolus injection of 1 mL solution into the pharynx through a nasal catheter. The subjects were unaware of the actual injection. Swallowing was identified by submental electromyographic (EMG) activity and visual observation of the characteristic laryngeal movement.[2] EMG activity was recorded from surface electrodes on the chin. The swallowing reflex was evaluated by the latency of response, which was timed from the injection to the onset of swallowing.[2] To measure dose-response effects of capsaicin on the swallowing reflex, patients received either vehicle or capsaicin (10^{-12} to 10^{-9} mol/mL). Studies on each concentration of capsaicin were done double-blind and randomised, with an interval of 2 min. The latency of response to vehicle was significantly longer in the patients than in the controls (8·1 [0-9] *vs* 1·0 [0-1] s, $p < 0.001$). The effect of capsaicin was dose-dependent (figure). No side-effects or unpleasant sensations were observed.

The addition of a low dose of capsaicin to liquid or food may stimulate the swallowing reflex and help to prevent aspiration pneumonia in the elderly.

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Differential response in a patient treated with Campath-1H monoclonal antibody for refractory non-Hodgkin lymphoma

SIR,—Lymphocyte-depleting monoclonal antibodies (Mabs) are increasingly used in patients with lymphomas, with rheumatoid arthritis, and in patients undergoing organ and bone marrow transplantation.[1-3] We are treating patients with refractory non-Hodgkin lymphoma (NHL) in a phase I/II multicentre study with Campath-1H. This humanised antibody is reactive to the CDw52 antigen expressed on most lymphoid cells, including malignant lymphoma cells. We report a patient with an interesting response to Campath-1H who illustrates some important issues associated with the use of Mab in patients with malignant disease.

A 50-year-old man was diagnosed in June, 1991, with a stage IVB intermediate grade NHL (diffuse mixed small and large cell NHL, International Working Formulation grade F). He had disease involving lymph nodes, spleen, and bone marrow. He was treated with six courses of CHOP (cyclophosphamide, doxorubicin,

vincristine, and prednisolone) and achieved partial remission with reduction in organomegaly and the degree of bone marrow infiltrate. He relapsed after 6 months and was treated with interferon-alfa to which he did not respond. Before entry into the Campath-1H study he developed progressive disease. The spleen was 20 cm palpable below the left costal margin. He also had extensive abdominal lymphadenopathy and circulating lymphoma cells. Bone marrow was heavily infiltrated (>80%) by lymphoma cells that strongly expressed CDw52 and also expressed CD5 and CD19. He received four weeks of Campath-1H 25 mg three times a week by intravenous infusion over 2 h, which was tolerated well with minor rigors and high temperature.

His spleen had become impalpable, although it was enlarged on computed tomography (CT). Circulating lymphoma cells were cleared from the blood. Bone marrow examination revealed a reduction in the malignant infiltrate (<5%, confirmed by immunophenotyping). However, abdominal CT showed progressive enlargement of lymph nodes. Bone marrow harvest four weeks after completing the course of Campath-1H preceded ex-vivo purging with Campath-1M (a rodent IgM directed at CD52). The unpurged marrow contained 16% CD5/CD19 positive cells, but these were reduced to under 0·4% after treatment with Campath-1M. We now plan to treat this patient with high-dose myeloablative chemoradiotherapy followed by autologous bone marrow rescue (ABMT).

We observed a differential pattern of response (good response in blood, bone marrow, and spleen but disease progression in lymph nodes), which probably indicates different accessibility of the Mab to tumour cells in various body compartments. Antibody therapy for nodal disease may best be given in the setting of minimum residual disease where nodal size is small and antibody accessibility may be increased. Mab therapy for bone marrow disease, however, seems promising. Although complete remission did not result, Campath-1H therapy was associated with substantial cytoreduction of lymphoma cells from bone marrow. Ex-vivo cytoreduction with Campath-1M marrow purging led to marrow clearance of significant lymphoma cells and permitted bone marrow harvesting and subsequent ABMT. The Mab was given with minimum adverse reaction and on an outpatient basis. Unlike regimens, such as miniBEAM, which are sometimes given to clear bone marrow disease before ABMT in patients with lymphoma, Campath-1H did not result in pancytopenia and our patient therefore did not require blood or platelet support. He also did not develop infection during Campath-1 therapy. Since Campath-1 is not reactive with the haemopoietic stem cells that repopulate the bone marrow after myeloablative chemoradiotherapy, its use before ABMT may also be preferable to chemotherapy which could damage stem cells and delay bone marrow regeneration.

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Cyclosporin absorption from microemulsion formulation in liver transplant recipient

SIR,—The absorption of oral cyclosporin depends on normal flow of bile into the proximal bowel and is impaired by cholestasis. In the early postoperative period after liver transplantation, patients with external biliary diversion (via a T-tube) fail to absorb the conventional oral cyclosporin solution Sandimmun,[1] and require parenteral infusion of the drug until normal bileflow to the gut is restored. In some patients, however, persistent cholestasis may warrant continued intravenous use, prolonging hospital stay and exposing the patient to a greater risk of severe side-effects, such as seizures.[2] The poor absorption of the conventional oral solution may be overcome by the development of oral formulations with improved absorption characteristics.[3] We have compared the pharmacokinetics of a new oral microemulsion formulation (Neoral) with the conventional oral solution in a 55-year-old female liver transplant recipient who had impaired absorption of drug due to a biliary stricture and who subsequently had acute allograft rejection. Trough blood cyclosporin concentrations, measured by monoclonal-antibody-based radioimmunoassay (Cyclo Trak-SP, Incstar), remained undetectable (below 25 μg/L) despite increasing doses of the conventional oral solution up to 8 mg/kg per day.

Throughout the pharmacokinetic study the patient was maintained on a continuous intravenous infusion of cyclosporin (0·8 mg/kg per day). The patient was given a test dose (5 mg/kg) of conventional cyclosporin capsules on the 4th day after beginning the infusion and given the same dose of the microemulsion in capsules 3 days later. Three steady-state blood cyclosporin concentrations measured on the day before both oral doses were within the range 220–250 μg/L. Blood samples for pharmacokinetic analyses were collected immediately before both oral doses and at 15 timed intervals over a 24 h period after each dose. Mealtimes were standardised after both oral formulations.

The timed blood samples collected after the two oral doses were randomised before assay. The area under the concentration versus time curves (AUC) was estimated by the trapezoidal rule and used to calculate bioavailability (F) from: $F = (\text{infusion rate})(C_{SS}^{-1})(\text{AUC})(\text{oral dose}^{-1})$, where C_{SS} is the average steady-state cyclosporin concentration during the infusion measured before the oral doses.

Despite poor absorption of cyclosporin from both oral formulations, bioavailability with the microemulsion (4·0%) was 8·5-fold greater than that with the conventional solution (0·47%). On the basis of these results, the cyclosporin infusion was discontinued and the patient stabilised on a microemulsion dose of 10 mg/kg daily. The mean of three trough blood cyclosporin concentrations measured during the subsequent week on this dose was 120 μg/L, and the patient was discharged home.

This new oral formulation may have considerable impact on the management of liver transplant recipients in reducing the requirement for intravenous cyclosporin with its attendant side-effects and in accelerating discharge from hospital.

We thank Sandoz for supplying the cyclosporin microemulsion capsules.

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Serum lipids and depression

SIR,—The paper by Mr Morgan and colleagues (Jan 9, p 75) linking low cholesterol and depression in elderly men prompted us to look at the relation between lipids and depression in the Helsinki Ageing Study, the baseline lipid values of which have been reported.[1] In this study of cohorts aged 75, 80, and 85 years (n = 621 with lipid determinations) the baseline assessments included standard lipid analyses, questionnaires, and a health examination. A questionnaire-based follow-up was done after a year. Depression was assessed by questionnaire (history of depression and Zung self-rating depression scale [SDS][2]) and by the examining physician's assessment of depression, severe depression being as defined in DSM-III. Data were analysed with BMDP statistical software.

Neither in men nor in women was baseline serum total or low-density-lipoprotein (LDL) cholesterol significantly associated with any baseline variables related to depression. Nor were serum triglycerides in men associated with these variables, although men with low high-density lipoprotein (HDL) cholesterol (<1·0 mmol/L) tended to have higher baseline SDS sum scores. In

are identified at a slightly later stage in disease progression. This idea is consistent with the difference in age (mean 24 vs 35 years) between our groups 1 and 2 (A-B vs C).

There is a further point of interest in the comparative subgroup of group 2 (1 previous primary melanoma and a normal naevus pattern). No second primary melanomas were seen in this group, whereas 0·026 would have been expected. This finding confirms those from studies of multiple primary melanomas that large numbers of naevi are a risk factor for second and subsequent primary tumours.[9] Among patients with previous melanomas the difference in age between those with atypical and normal naevus patterns 35 [16–63] *vs* 54 [28–79] years) is significant ($p < 0·01$); this finding suggests that patients with an atypical naevus pattern have either genetic or early life environmental factors that accelerate the development of melanoma. It is tempting to speculate that this factor may be early childhood sun exposure, which not only causes development of large numbers of naevi[11,12] but also acts as an initiating agent for later development of melanoma.

Patients and their relatives in category D are clearly at greatly increased risk of a first or subsequent primary tumour and need life-time surveillance. The lack of a significant difference in risk between combined categories A–C and category D may reflect the small numbers and thus wide confidence intervals in group D.

Photographic recording of naevi and use of these photographs to detect change suggesting development of melanoma has been essential in this study. Only one of the surveillance-detected melanomas was suspected by the patient. The value of surveillance is also supported by the thickness of the tumours at detection; only 2 were thicker than 1·5 mm and both of these, on review of photographs, showed a greater degree of inflammation at 6 months before clinical suspicion and excision than any of the thinner melanomas. Although we and others have previously described inflammation as a clinical characteristic of benign but atypical naevi, we would recommend caution in dismissing persistently inflamed melanocytic lesions as benign, and suggest excision.

In-situ or level-1 melanomas were not included in calculations of relative risk, since it is not clear whether all in-situ lesions progress to invasive melanomas. The lack of an appropriate animal model makes it impossible to investigate this question further at present. If even a proportion of in-situ lesions are progressive, our calculations are an underestimate of the magnitude of the relative risk.

The incidence of melanoma was 709 per 100 000 in category A–C individuals and 4429 per 100 000 in category D; the rates in the general Scottish population range from 5–10 per 100 000 for the relevant age groups. By comparison, current incidence rates for breast carcinoma and cervical cancer, in the age ranges that merit specifically funded screening programmes, are 224 and 22·4 per 100 000. There is, therefore, a strong case for funding of melanoma surveillance programmes or clinics aimed at facilitating early diagnosis for individuals with atypical naevi, especially given that, despite intensive research, there have been few advances in the management of melanoma that has spread beyond the primary site.

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Long-term remission of intractable systemic vasculitis with monoclonal antibody therapy

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Monoclonal antibodies that target T cells offer an alternative to conventional immunosuppressive drugs in the management of autoimmune disease. "Humanisation" of such monoclonal antibodies makes their clinical use less likely to be prone to the risk of cross-species sensitisation than treatment with rodent antibodies. We describe humanised monoclonal antibody therapy in four patients with severe systemic vasculitis unresponsive to immunosuppressive drugs. Substantial and sustained benefit was seen in three of the four patients, although one of these three patients developed anti-idiotypic antibodies that had to be removed by plasma exchange.

Lancet 1993; **341**: 1620–22.

A wide range of monoclonal antibodies (mAbs) that react with T cells have been examined as possible therapeutic agents in experimental autoimmune disease.[1] However, experience with the use of mAbs in man for such conditions is limited. We have reported successful use of a combination of two mAbs to treat a patient with autoimmune systemic vasculitis.[2] Here we report the use of such mAbs to treat three further patients with similar vasculitis and describe the progress of the first patient during the 42 months since he received the original treatment regimen.

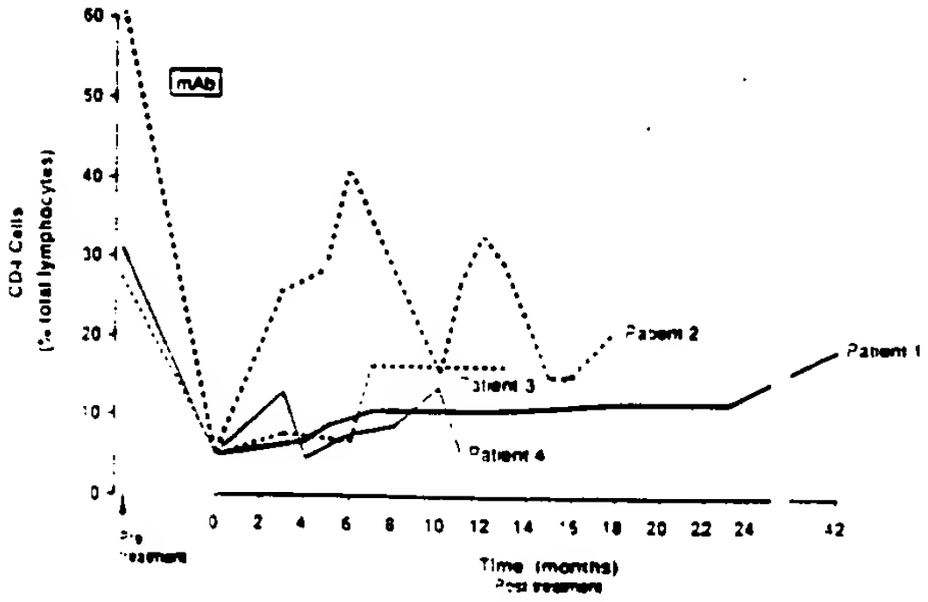
CLINICAL AND LABORATORY DETAILS OF PATIENTS

Patient	Diagnosis	Clinical involvement	Biopsies showing abnormal cell infiltrates	Dose of mAb, in mg (days)		Remission (months)	Need for further mAb treatment
				Anti-CDw52	Anti-CD4		
1	Microscopic polyarteritis	Vasculitic rash, arthritis, scleritis, pleuritis, pericarditis	Vasculitic rash, nerve, artery	2 (8)	20 (12)	42	No
2	Microscopic polyarteritis	Vasculitic rash, arthritis, pleuritis pericarditis, acalculous cholecystitis	Synovium (without vessel-wall necrosis)	12 (5)	20 (5)	7, 3, 12	Yes
3	Sjogren's syndrome	Vasculitic rash, arthritis, pleuritis, pancreatitis, optic neuritis, nephritis	Vasculitic rash, lung, kidney	40 (5)		13	No
4	Behcet's disease	Vasculitic rash, arthritis	Vein, lung	10 (5)	20 (10)	3	No

All patients were refractory to prednisone, cyclophosphamide, azathioprine, cyclosporin A. In addition, patients 1, 2, and 4 had failed treatment with plasma exchange and high-dose pooled intravenous immunoglobulin, and patient 4 had received total lymph node irradiation before referral.

All four patients had life-threatening disease and had been refractory to conventional immunosuppressive agents. Details of the patients' clinical presentations and laboratory investigations are shown in the table. All patients were treated with CAMPATH-1H, a "humanised" antilymphocyte mAb that recognises the CDw52 antigen within the lymphocyte population. The major effect of CAMPATH-1H appears to be on T-cell numbers and hence T-cell function. Depletion of CD4 and CD8 subpopulations has been reported after administration of CAMPATH-1H in man.[3] In addition, patient 1 was given YNB 46·1,[4] a rat IgG2b that can interfere with the function of CD4 antigen, and patients 2 and 4 received hIgG1CD4, a humanised monoclonal anti-CD4 antibody. The mAbs were administered intravenously in doses up to 40 mg daily and their depleting effect on circulating lymphocyte populations* was determined as described previously.[3] Antiglobulin and anti-idiotype responses to these antibodies were assessed by double-capture enzyme-linked immunosorbent assays.[4] The assay for antibodies to CAMPATH-1H was capable of detecting 2 µg/mL of polyclonal goat anti-human IgG (Sigma) and 10 ng/mL of monoclonal anti-idiotype antibody YID 13·9 (which recognises the CAMPATH-1H idiotype). The assay for antibodies to anti-CD4 mAbs could detect 250 ng/mL of the anti-rat IgG2b mAb NORIG 7·16.

Only patient 2 made an antiglobulin response against CAMPATH-1H. This was a pure anti-idiotype response, which was first detected after the failure of a third course of therapy and reached a titre equivalent to 16 µg/mL of YID 13·9. Antiglobulins became undetectable after plasma exchange, but were boosted within a week of the fourth course of treatment to a concentration equivalent to 120 µg/mL of YID 13·9.* There was prolonged depletion of CD4 cells in all patients treated with mAbs (figure), but only patient 4 developed an opportunistic infection—oral candidiasis—which responded to appropriate treatment.



Effect of CAMPATH-1H on peripheral CD4 cells.

There was substantial depletion of CD4 cells with mAb treatment when measured as % total circulating lymphocytes (normal range greater than 25%). In patient 2, a rise in CD4 cells occurred at times of relapse. Recovery of CD4 counts to $0·2 \times 10^9$/L or greater took place after two (patient 1), twelve (patient 2), or eleven months (patients 3 and 4).

The beneficial immunoregulatory effect of mAb therapy was still present after a year in three patients and after three and a half years in one of these patients. Although CD4 counts fell after mAb treatment in the fourth patient, persistence of raised circulating interferon gamma and C-reactive protein concentrations suggested that these inflammatory indices were independent of the CDw52-positive population of lymphocytes. Subsequent treatment of the fourth patient with thalidomide, which inhibits interferon-gamma-mediated tumour necrosis factor release from monocytes,[5] has secured long-term disease control.

Early experience with mAb therapy suggested that certain patients with intractable forms of vasculitis could respond to treatment directed at T cells rather than at B cells and their products.[2] All the patients described here had disease characterised by vasculitis, with biopsy evidence of T-cell infiltrates in and around vessels and in the interstitial tissues of affected organs, lack of circulating vasculitis-associated antineutrophil cytoplasm antibody, and resistance to conventional immunosuppression. However, it is uncertain whether T cells mediated directly tissue injury in our patients, since, even with repeated biopsy, it was difficult to identify vessel-wall necrosis juxtaposed to T-cell infiltrates. Nevertheless, experimental models suggest that T cells may have some direct involvement in vessel damage.[6] Furthermore, all four patients responded rapidly to antibody treatment, the promptness of improvement (usually within 72 h) suggesting that cell-mediated rather than antibody-mediated mechanisms were affected predominantly.

Despite "humanisation", an anti-idiotypic antibody response was detected in the second patient after two courses of CAMPATH-1H. The humanised anti-CD4 antibody (hIgG1CD4) was not available at the time this patient first needed treatment. Whether development of the anti-idiotypic response can be avoided in future by the early combination of anti-CD4 antibody with CAMPATH-1H remains to be evaluated. Experimental studies have shown that combination therapy can give better tolerance than either mAb used alone,[7] and better control of the progression of an established autoimmune response.[8] We believe such a combination strategy may offer substantial benefit in some patients. However, CAMPATH-1H alone was effective long-term in our third patient when given in a sufficient dose. Whether reprogramming of the immune system by mAb treatment can regularly provide sustained effects independent of other treatment, or whether, as in our

*Figures showing effect of CAMPATH-1H on circulating lymphocyte counts and renal and lung function in patient 3, and development of anti-idiotypic responses in patient 2, are available on request from *The Lancet*.

second patient, it restores sensitivity to low doses of conventional immunosuppressive drugs (unpublished) remains to be seen. Further studies to explore use of these mAbs in other autoimmune diseases seem warranted.

This work was supported by the Medical Research Council, the Wellcome Trust, the Wellcome Foundation, and the Gilman Foundation. We thank Dr A. J. Crisp, Professor M. J. Brown, and Dr A. M. Denman for allowing us to study their patients. We also thank Ms Helen Waller for technical assistance, and Dr J. Phillips and the staff of the therapeutic antibody centre for production of antibodies. C. M. L. is a Wellcome Reader in the School of Clinical Medicine. CAMPATH-1H is a trademark of the Wellcome Foundation.

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T-cell-mediated response in Dupuytren's disease

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The cause of Dupuytren's disease is unknown, but inflammatory cells might have a role. Enzymatic digestion of diseased tissue permits identification and immunofluorescent labelling of a cell subset displaying inflammatory cell morphology. Cytofluorimetry of this cell population demonstrated the presence of CD3-positive lymphocytes and expression of major histocompatibility complex (MHC) class II proteins. These results raise the possibility that Dupuytren's disease is a T-cell-mediated autoimmune disorder. The development of medical treatment on this basis may reduce the need for surgery, with its associated morbidity and high recurrence rates.

Lancet 1993; **341**: 1622–23.

Individuals with Dupuytren's disease have a genetically-determined predisposition to the condition.[1] The precise aetiology remains unclear but fibroblasts and myofibroblasts are thought to have a central role,[2] and superoxide free radicals might be the stimulus to myofibroblast proliferation.[3] The condition may also occur in association with various other medical disorders.[4,5] The prevalence and clinical significance of inflammatory cells in diseased tissue is controversial.[6] Baird et al[7] have demonstrated cytokine expression in tissue from Dupuytren patients, and certain inflammatory cells are known to be potential sources of these intercellular signalling molecules. We have examined inflammatory cells in tissue from patient's with Dupuytren's disease by flow cytometry of digested specimens to reduce sampling errors in histological examination of this heterogeneous tissue.

Specimens of subcutaneous tissue were obtained from 13 male and 1 female (aged 43–77 years, mean 63) Dupuytren's patients undergoing palmar fasciectomy. Control tissue was obtained by combining palmar fascia specimens excised from 3 male and 3 female (aged 38–67, mean 58) patients having carpal tunnel decompression. These specimens were pooled to obtain adequate cell numbers for analysis. All operations were done under regional anaesthesia with exsanguination of the limb. Tissue was diced into 2 mm fragments and digested at 37°C in collagenase 0·1%/DNAse 0·01% for 3 h followed by trypsin 0·05%/edetic acid 0·02% for 1 h. Filtration yielded a cell suspension in which residual enzyme activity was neutralised by washing and resuspending cells in Dulbecco's modified eagle medium containing 10% fetal calf serum (Gibco). Cells were plated out at a maximum initial density of 5×10^6 cells per dish and incubated overnight to allow separation of adherent cells (fibroblasts and macrophages) from inflammatory cells. This procedure also allowed regeneration of cell surface markers depleted by enzymatic activity. Non-adherent cells were harvested and samples of $2{\cdot}0–3{\cdot}0 \times 10^5$ cells were resuspended in 200 µl medium, then labelled with fluorescent monoclonal antibodies designed for flow cytometry (Dako). The panel of paired fluorescent antibody combinations used and the inflammatory cell subsets that they recognise were: CD45/14 (pan-leucocyte/monocyte); CD3/19 (pan T-cell/pan B-cell); CD3/4 (pan T-cell/T-helper cell); CD3/8 (pan T/cytotoxic T-cell); HLA-DR/CD3 (activated cells/pan-T); and CD16 + 56 (natural killer [NK] cells). Cells were labelled at room temperature for 10 min with 10 µl of neat antibody, washed with phosphate-buffered saline, fixed with 1% paraformaldehyde, and fluorescence was measured with the Lysys II programme on a Becton Dickinson FACScan flow cytometer. Dedicated software permitted statistical analysis of the data obtained. Cytospin preparations of both adherent and non-adherent cells were stained with Giemsa's stain for examination by light microscpy.

Light microscopy of adherent cells showed a homogeneous subpopulation of relatively large cells with foamy cytoplasm. By striking contrast the non-adherent subpopulation was heterogeneous, with many small, darkly-stained cells having the appearance of lymphocytes. The table shows the results from flow cytometry of non-adherent cells. 15–30% (mean 25%) of all Dupuytren cells in the lymphocyte gate were CD3-positive T-lymphocytes, compared with less than 0·5% of non-adherent cells from normal fascia. HLA-DR antigen was detected in 15–41% (mean 27%) of all gated cells from Dupuytren's patients, compared with 1·2% of controls. All other markers were expressed infrequently on Dupuytren cells, indicating a low prevalence of CD4 T-helper cells (1·6–4·3%) and CD8 cytotoxic T-cells (2·7–8·0%). Not shown in the table (because of uniformly low-labelling frequency) are CD16 + 56 NK cells (< 1–1·1%), CD19 B-lymphocytes (< 1–5·0%), and CD14 monocytes (< 1%). Very low frequencies of antibody labelling were also demonstrated by the pooled control fascia cells (less than 1·5% in each case).

Tissue in Dupuytren's disease contains substantial numbers of CD3-positive T-cells, suggesting that they are important mediators in the pathogenesis of this condition. The low prevalence of CD4, CD8, and CD16 + 56 antigens indicates that most of these cells may belong to a further

Continuous ambulatory subcutaneous desmopressin infusion in patient with diabetes insipidus

SIR,—A 23-year-old woman was admitted with loss of control of her diabetes insipidus, with a daily diuresis of 3500–7000 mL. From age 2, her diabetes insipidus had been adequately regulated with desmopressin intranasally but this method of administration had recently proved insufficient. An explanation for this acquired insensitivity could not be found. The addition of carbamazepine and later alteration to an oral form of desmopressin gave no improvement. With subcutaneous administration of desmopressin three times daily, the diuresis varied from 600 to 4500 mL, which the patient experienced as very unpleasant. The best result was achieved with intramuscular injections, but the patient did not want to administer these herself. We therefore decided to use continuous subcutaneous administration, such as is used in the treatment of patients with diabetes mellitus (Nordisk infuser MK 2). The sterilised ampoules were filled with desmopressin by the pharmacist (1·714 μg = 2·5 mL solution). The best results were achieved when each ampoule was used for no longer than 24 h. The patient was quickly able to adjust the infusion speed on the basis of diuresis and thirst, to give a daily diuresis of 1000–2500 mL. This system has now operated satisfactorily for 2 years without complications. The patient has even had her first baby in this period.

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Failure of subcutaneous vasopressin in diagnosis of central diabetes insipidus

SIR,—We report a patient in whom the diagnosis of central diabetes insipidus could be made only after the administration of vasopressin intravenously, not subcutaneously, as is recommended. A 34-year-old woman had haemorraghic shock due to massive upper gastrointestinal bleeding, followed by septic shock, during chronic illness that resulted from acute pancreatitis with multiple organ failure. 2 days after the bleeding episode the patient had polyuria (up to 800 mL/h) and hypernatraemia (up to 157 mmol/L) with dilute urine (238 mosmol/kg). Vasopressin 5 U was given subcutaneously,[1-3] but urinary output and osmolality did not change. After several hours the test was repeated with intravenous vasopressin 5 U, resulting in an increase of urine osmolality from 289 to 448 mosmol/kg, and a dramatic drop in urinary output, thereby establishing the diagnosis of central diabetes insipidus. At that time the patient was very oedematous and was being treated in intensive care with vasoactive drugs, among others. Thus the subcutaneous vasopressin test can lead to the erroneous diagnosis of nephrogenic diabetes insipidus in the critically ill patient.

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Adverse reactions to Campath-1H monoclonal antibody

SIR,—Dr Lim and colleagues (Feb 13, p 432) report a case of non-Hodgkin lymphoma resistant to first-line chemotherapy and interferon that was treated with the humanised antibody to the CDw52 antigen (Campath-1H) with minimal adverse reactions. They report a poor response to this immunotherapy in lymph node masses, but good clearance from the bone marrow, blood, and spleen, and plan to consolidate this with an autologous bone marrow transplant.

We also report a differential response between lymph nodes and bone marrow seen in four consecutive patients treated for up to 9 months with higher doses of Campath-1H (up to 80 mg three times a week) than used by Lim and colleagues. Three out of four patients with bone marrow involvement have had a complete response in the bone marrow, but axillary and cervical node regression was seen in one patient who had no response in the abdomino-pelvic nodes. However, several quite serious adverse reactions (anaphylaxis, neutropenia, and opportunistic infections) occurred at different doses. Prolonged bone marrow hypoplasia was seen in two patients given Campath-1H at doses ranging from 8 to 80 mg three times a week over 6–9 months. The first patient received 11 doses of 80 mg given three times a week. An almost total marrow aplasia developed with a nadir white blood count (WBC) of 0·12 × 10⁹/L, platelets less than 15 × 10⁹/L, and a neutrophil count of 0·07 × 10⁹/L. With intensive supportive care his marrow is slowly recovering, and 4 months after stopping treatment the WBC is 1·9 × 10⁹/L with 1·39 × 10⁹/L neutrophils. Axillary and cervical lymph node masses have resolved further off treatment, whereas pelvic ones have progressed in size. A second patient also had hypoplasia of the marrow, but Campath-1H treatment was stopped immediately (cellularity of trephine was 25%), and the marrow recovered within two months to a cellularity of 80%.

Our patients also developed severe infections such as generalised herpes zoster, despite neutrophil counts greater than 1 × 10⁹/L, and septicaemia including one episode caused by *Listeria monocytogenes*. A number of patients had rigors, one of which was associated with hypotension needing resuscitation with adrenaline. These reactions were idiosyncratic in nature, in as much as they were not necessarily related to the first dose and did not recur after rechallenge with Campath-1H. We have not seen the development of neutralising antibodies to Campath-1H, but this is clearly another potential problem. One patient experienced a severe anaphylactoid reaction to a dose of 25 mg of Campath-1H, but continued in complete remission in the bone marrow on 8 mg of Campath-1H three times a week for 3 months, and after consolidative radiotherapy has received an allogeneic bone marrow transplant, and is now in full remission 6 months later.

In conclusion, we agree with Lim and colleagues that Campath-1H is an exciting new treatment for resistant non-Hodgkin lymphoma, but caution against underestimating its potential toxicity. The differential response in marrow and lymph nodes needs further investigation by studying the biodistribution of Campath-1H and its relation to access to the patients' immune effector mechanisms, such as antibody-dependent cellular cytotoxicity. The current multicentre phase II study with Wellcome Research Laboratories may provide answers to some of these questions in a much larger group of patients.

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Fetal development after chorionic villus sampling

SIR,—Professor Rodeck (Feb 20, p 468) made several important recommendations to minimise the risk of limb reduction defects after chorionic villus sampling (CVS), but also recommended that further interventions in early pregnancy, such as amniocentesis before 14 weeks, should not be routinely introduced before full evaluation. We agree that scientific evaluation is needed, but suggest that possible problems of early amniocentesis are more similar to those of the safe standard amniocentesis[1] than to those of CVS.

We studied early amniocentesis done between weeks 12 and 14 of gestation (40% were from week 11 + 1 to week 12 + 6 [week 12 and 13]) in 1500 women. After obligatory genetic counselling, about 8 mL of fluid were taken under ultrasonographic vision. In 99·7%, karyotyping was successful with a mean culture time of 14·5 days. Up to week 24, the complication rate (spontaneous abortion, amniotic fluid leakage, or vaginal bleeding) was 1·1% (*vs* 0·7% in 6000 women who had standard amniocentesis). The total

EFFECTOR FUNCTIONS OF A MONOCLONAL AGLYCOSYLATED MOUSE IgG2a: BINDING AND ACTIVATION OF COMPLEMENT COMPONENT C1 AND INTERACTION WITH HUMAN MONOCYTE Fc RECEPTOR

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Received 20 *July* 1984; *accepted* 10 *September* 1984.

Abstract—Aglycosylated monoclonal anti-DNP mouse IgG2a produced in the presence of tunicamycin was compared with the native monoclonal IgG2a with respect to its ability to interact with the first component of complement, C1, and to compete with human IgG for binding to human monocyte Fc receptors. The aglycosylated IgG2a was found to bind subcomponent C1q with an equivalent capacity to the native IgG2a, but the dissociation constant was found to be increased three-fold. When activation of C1 by the glycosylated and aglycosylated IgG2a was compared, the rate of C1 activation by the aglycosylated IgG2a was reduced approximately three-fold. In contrast aglycosylation was accompanied by a large decrease ($\geq$50-fold) in the apparent binding constant of monomeric IgG2a to human monocytes. The data suggest that the aglycosylated IgG2a has a structure which differs in the C_H2 domain from the native IgG2a, and that the heterogeneous *N*-linked oligosaccharides of this monoclonal IgG2a which occur at a conserved position in the C_H2 domain play a role in maintaining the integrity of its monocyte-binding site. This lack of monocyte binding may result either from a localized conformational change occurring in a single C_H2 domain or from an alteration in the C_H2–C_H2 cross-domain architecture which is normally structured by a pair of opposing and interacting oligosaccharides. The minimal changes in C1q binding and C1 activation suggest that the oligosaccharides are, at most, indirectly involved in these events.

INTRODUCTION

An integral feature of all normal IgG class antibodies is the *N*-linked oligosaccharides in the C_H2 domain. Analyses of the structural and functional aspects of the *N*-linked oligosaccharides are of biological interest for four main reasons: (1) the glycosylation of the C_H2 domain has been conserved throughout evolution, suggesting an important role for the oligosaccharides, (2) the combinatorial association of two heavy chains places two oligosaccharide units in direct contact with each other from which it follows that (3) the immunoglobulin molecule is a model for both specific protein–carbohydrate and carbohydrate–carbohydrate interactions, and (4) the immunoglobulin molecule serves as a model system for the analysis of oligosaccharide heterogeneity (Rademacher and Dwek, 1984; Rademacher *et al.*, 1982).

Crystallographic studies on immunoglobulin Fc fragments (Diesenhofer, 1981; Sutton and Phillips, 1983) have shown that, unlike other immunoglobulin domains, the two C_H2 domains do not form extensive lateral associations. The resultant interstitial region is filled by the inclusion of the oligosaccharide side chains attached to Asn-297 on each heavy chain such that the carbohydrates form a bridge across the domains. The $\alpha(1-6)$ arms of the biantennary complex oligosaccharides interact with the protein and the $\alpha(1-3)$ arms of the two oligosaccharides form the bridge.

Possible roles for the oligosaccharides could include both a structural and/or functional one. In the former the oligosaccharides would exist as spacers between the two C_H2 domains, thereby imparting

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stability or protection (e.g. from proteolysis). However, the presence of oligosaccharide heterogeneity could also increase the functional diversity of the immunoglobulin molecule with the oligosaccharides acting as specific ligands for recognition by receptors.

This paper describes the production of a monoclonal aglycosylated IgG2a and the purification of this IgG2a to homogeneity. We then compare the interaction of native and aglycosylated IgG2a with the first component of complement C1. This is achieved by quantitative comparison of the binding affinity and capacity for the subcomponent C1q alone, and by comparison of the rates of activation of the entire C1 complex. The results allow a quantitative description of the effect of aglycosylation on the binding and activation of C1. We also compare the interaction of native and aglycosylated mouse IgG2a with human monocyte Fc receptor and discuss the role that the oligosaccharide may be playing in immunoglobulin structure and function.

MATERIALS AND METHODS

Materials

All culture media were obtained from Gibco. Ficoll 400 and CNBr-activated Sepharose 4B were purchased from Pharmacia Fine Chemicals. Metrizoic acid [sodium salt, 32.8% (w/v) aq. solution], tunicamycin and rabbit anti-bovine IgG were obtained from Sigma Chemical Co. L-[4,5-^{3}H]leucine (197 Ci/mmole) and D-[1-^{14}C]glucosamine HCl (57.9 mCi/mmole) were obtained from Amersham International. [^{125}I]-C1 was generously provided by B. Gorick and N. R. Hughes-Jones (ARC, Cambridge). The hybridoma cell line K3 was made available by Dr B. A. Askonas (N.I.M.R., Mill Hill, U.K.).

Immunoglobulin

Pooled human IgG was isolated using ammonium sulphate precipitation, ion-exchange and gel-filtration chromatography (Hudson and Hay, 1980) and labelled with ^{125}I using immobilised lactoperoxidase/glucose oxidase (Bio-Rad) to a level of ~0.02 I/mole.

The IgG2a-producing hybridoma (K3) was grown as an ascitic tumour in pristane-primed (BALB/c × CBA) F_1 mice. Viable hybridoma cells were purified from ascitic fluid on a discontinuous Ficoll/Metrizoate density gradient (Davidson and Parish, 1975) by recovery from the interface. The purified hybridoma cells were seeded at a density of 2×10^5 cells/ml, and subcultured every 3 days as necessary in Falcon 3028 tissue culture flasks containing RPMI 1640/10% heat-inactivated foetal calf serum (FCS)/penicillin (100 units/ml)/streptomycin (100 μg/ml)/2-mercaptoethanol (25 μM)/glutamine (2 mM).

Aglycosylated IgG2a was produced by suspending cells from the above cultures at 2×10^6 cells/ml in medium containing 2 μg/ml tunicamycin. The cells were incubated in this medium at 37°C 5% CO_2 for 4 hr, which control experiments had determined to be sufficient time for all remaining glycosylated IgG2a to be secreted. The cells were then centrifuged at 160 *g* for 10 min, the supernatants removed, and the cells washed twice with fresh tunicamycin-containing medium, and resuspended at 2×10^6 cells/ml. They were then re-cultured for 3 days before removing the aglycosylated IgG2a-containing supernatant. The supernatants were concentrated 10-fold by ultrafiltration using an Amicon PM30 membrane, dialysed vs PBS/EDTA, and any precipitated material removed by centrifugation. The supernatant, with [^{3}H]leucine IgG2a added to act as a tracer, was then applied to a DNP–lysine–Sepharose column (5 × 1.4 cm) in PBS/EDTA, and unbound material washed through with the same buffer. Bound protein was eluted with 50 mM DNP–glycine, pH 7.2. After concentration, this protein was applied to a Sephacryl S-200 column (140 × 1.6 cm) in PBS/EDTA to purify the product further and remove excess DNP–glycine. Removal of bovine IgG derived from the foetal calf serum was by passage of the IgG fraction through an anti-bovine IgG–Sepharose column. Remaining traces of bound DNP–glycine were removed by exchange dialysis against dinitrophenol in PBS at pH 7.2 followed by ion-exchange chromatography on Dowex 1 × 8 (-400).

Tracer [^{3}H]leucine IgG was produced in culture using leucine-deficient RPMI 1640 (Gibco, select amine kit). Cells were incubated in the modified medium containing tunicamycin for 4 hr at 37°C/5% CO_2, washed and resuspended in fresh modified medium followed by the addition of radiolabelled amino acid (10 μCi/ml) and incubated for a further 30 hr prior to isolation of the IgG as above.

In order to determine the optimal amount of tunicamycin, cells (2×10^6 cells/ml, 0.5 ml) with varying amounts of tunicamycin (added from a stock solution in 5 mM NaOH/165 mM NaCl) were incubated for 3.5 hr at 37°C/5% CO_2 in Falcon 3033 tissue culture tubes. 2 μCi/ml L-[4.5-^{3}H]leucine and 1 μCi/ml D-[1-^{14}C]glucosamine hydrochloride were then added and incubation continued for 36 hr. After centrifugation the supernatants were removed and the labelled IgG isolated on columns of DNP–lysine–Sepharose. After washing with PBS the IgG was eluted with 50 mM DNP–glycine, pH 7.2, which was subsequently removed on a Dowex 1 × 8 (-400) column equilibrated in PBS.

IgG samples were analysed on a discontinuous SDS–PAGE. Fluorography was performed using ENHANCE (New England Nuclear) and Kodak X-Omat AR film. Proteins were visualized by staining with Coomassie blue.

Binding of IgG2a to human monocyte Fc receptors

Human mononuclear cells were isolated from freshly-drawn heparinized blood by use of lymphocyte separation medium (Flow). They were then

washed with RPMI 1640 10% FCS and incubated in plastic petri dishes at 37°C 5% O_2 for 45 min. The adherent monocytes were harvested by washing in 1:1 BSS [−Ca, −Mg]:EDTA (10 m*M*):RPMI 1640 10% FCS. Cells were then suspended at 10^7 ml in BSS [−Ca, −Mg]/BSA (0.2%)/NaN_3 (0.1%) and 2 vols of suspension was added to 1 vol of [^{125}I]-human IgG (4 n*M*), BSS [−Ca, −Mg]/BSA (0.2%)/NaN_3 (0.1%) and 1 vol of test IgG2a solution (various dilutions) and incubated for 2 hr at 37°C. Both the iodinated IgG and the test IgG2a stock solutions were centrifuged (100,000 *g* for 30 min) before use to remove aggregates. The monocytes were resuspended and duplicate aliquots were layered over 1 vol Versiluble F50 (Alfa) which had in turn been layered over 1 vol of 1:2 lymphocyte separation medium:BSS in siliconized tubes. The monocytes with bound IgG were separated from unbound IgG by centrifuging them through the Versiluble phase. The tubes were then frozen and snapped to separate the pellet and supernatant fractions, and the amount of [^{125}I]-IgG in each was determined. As well as varying amounts of test IgG, the following controls were also included: (1) [^{125}I]-IgG alone, to give the initial binding; and (2) a large excess of unlabelled IgG, to give the non-specific background binding.

Protein in the stock IgG samples after ultracentrifugation was determined by the Bio-Rad dye-binding protein assay (micro version) using dilutions of an IgG sample of known concn to construct the standard curve.

C1q binding

This was performed as described previously (Leatherbarrow and Dwek, 1984) except that the final concn of IgG2a/DNP–Affigel 701 was 0.6% (v/v) and the total assay vol was 100 µl. The data were analysed using a FORTRAN non-linear regression program.

C1 activation

This assay followed essentially the C1 activation assay of Folkerd *et al.* (1980) but used IgG·DNP–Affigel 701 to activate the C1. The supernatant and pellet fractions were analysed by SDS–PAGE using 10% acrylamide gels. Positions of the labelled protein bands were determined by autoradiography. The intensity of each band was estimated by densitometry of the developed film and the percentage activation expressed as:

$$\frac{(\bar{C1r}[H] + \bar{C1s}[H] + (\bar{C1r}[L])}{(C1r + C1s) + (\bar{C1r}[H] + \bar{C1s}[H] + (\bar{C1r}[L])} \times 100\%$$

where [H] and [L] denote the heavy and light chains of C1r or C1s produced after activation (denoted by a bar) which is due to the proteolytic cleavage.

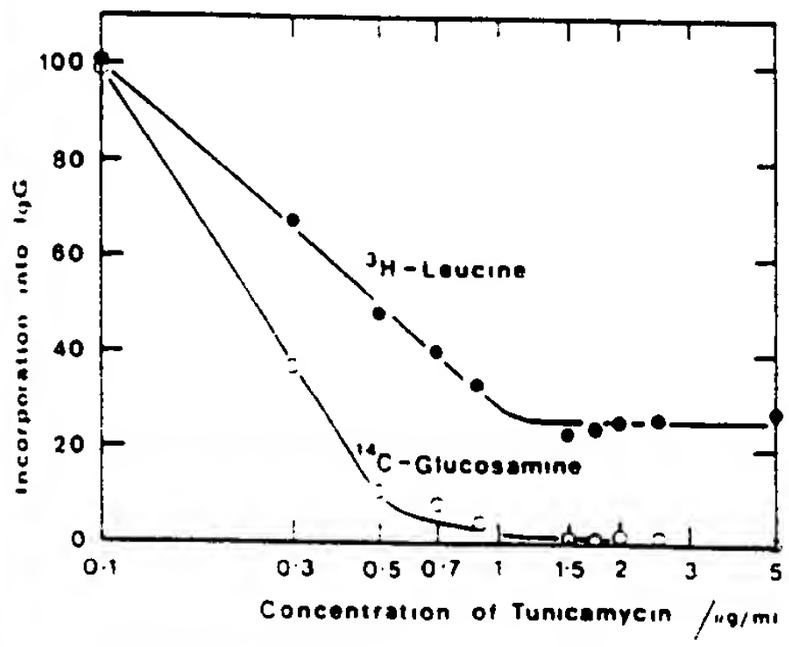

Fig. 1. Effect of tunicamycin on the incorporation of [^{3}H]leucine (●) and [^{14}C]glucosamine (○) into IgG2a isolated from culture supernatants. The results are expressed as the percentage incorporated in the absence of tunicamycin.

RESULTS

Effect of tunicamycin on IgG2a secretion and glycosylation

The effect of increasing tunicamycin concn on the incorporation of [^{3}H]leucine and [^{14}C]glucosamine into IgG2a produced by the hybridoma cell line K3 is shown in Fig. 1. Tunicamycin inhibits the glycosylation of the immunoglobulin in a dose-dependent manner, with complete inhibition occurring at > 1.5 µg/ml tunicamycin. These results are in accordance with previous findings where it has been found that, in contrast to other types of immunoglobulin, aglycosylated IgG is still secreted from the cells, albeit at a reduced rate (Hickman *et al.*, 1977; Hickman and Kornfeld, 1978; Blatt and Haimovich, 1981; Sidman, 1981). It is interesting to note here (see Fig. 1) that when the protein is completely aglycosylated no further inhibition of the IgG2a secretion is observed. Consequently a slight excess of tunicamycin (to ensure complete aglycosylation) was used (2 µg/ml) in the experiments with the K3 cell line.

Purification of aglycosylated IgG2a from culture supernatants

A total of 2.5 l of aglycosylated IgG2a-containing culture supernatant was produced as described above. The concn of mouse IgG2a in this supernatant was estimated to be 1–2 µg/ml by radial immunodiffusion. Glycosylated IgG2a was also produced as a control from parallel cell cultures in the absence of tunicamycin. Gel filtration of the eluate from the DNP–lysine–Sepharose column showed a single radioactive peak at $M_r \sim 150$ kilodaltons. Monitoring at 280 nm revealed two peaks, however, one eluting with the void vol, and the second at the position of the radioactive peak and corresponding to that of authentic IgG2a ($M_r \sim 150$ kilodaltons). The material eluting in the void vol did not contain IgG, and consisted of components of extremely high apparent mol. wt which did not enter a 5% acrylamide stacking gel. By criteria of reaction with anti-bovine IgG antisera and from the SDS gel profiles it was found that in addition to the mouse IgG2a the

EXHIBIT B-10

MRC/WELLCOME THERAPEUTIC ANTIBODY CENTRE

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PROGRESS REPORT

(1 May 1990 to 31 Dec 1990)

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Clinical results

Obviously, there are not yet any clinical data from antibodies produced in the CHO. However, over Christmas a patient with severe autoimmune vasculitis was successfully treated with CAMPATH-1H from our previous stocks. She had crippling joint disease due to lymphocyte infiltration and required large amounts of opiates to control the pain. Following 10 days of treatment there was a marked clinical improvement, lymphocytes were cleared, opiates could be withdrawn and the patient was able to start walking again. We have yet to see whether this dramatic recovery will be sustained or whether more antibody will be required. This result was very important to us because the CAMPATH-1H had been produced in CHO (chinese hamster ovary) cells rather than the rat myeloma cells as before. The CHO cells offer increased productivity and so are being used by Wellcome and by us for most future antibodies. However, there had been some doubt whether the antibody produced would be as effective in vivo. Therefore the successful treatment of this first patient has been very encouraging to all of us.